CHARACTERIZATION OF RESISTANCE TO BLACK SPOT DISEASE OF *ROSA* SPP.

A Dissertation

by

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ABSTRACT

Black spot disease (BSD), caused by the fungus *Diplocarpon rosae* Wolf, is one of the most serious diseases of garden roses. Both complete (vertical) resistance conditioned by dominant *Rdr* genes and partial (horizontal) resistance (PR) conditioned by multiple genes have been described. The use of resistant rose cultivars would reduce the demand of agrochemical application.

The characterization of 16 genotypes using two laboratory assays, the detached leaf assay (DLA) and the whole plant inoculation (WPI) approach, indicated that these were well correlated. Thus either method could be used to assess the resistance of the plants to the BSD. Fifteen diploid hybrid populations from 10 parents segregating for black spot partial (horizontal) resistance were assessed for black spot resistance by quantifying by the percentage of the leaf area with symptoms (LAS) and lesion length (LL) measured by the diameter of the largest lesion in detached leaf assays. Nine of these populations were also evaluated in field trials by rating the incidence of damage due to the fungal infection. The narrow sense heritability of partial resistance to black spot as measured by LAS and LL data of DLA was estimated from 0.3 to 0.4 when calculated with a genetic variance analysis and from 0.7 to 0.9 when generated from mid-parent offspring regression. In the field assessments, the second year assessments were better than the assessments done the first year due to higher and more uniform inoculum levels which minimized problems with escapes. In general there was no or just low correlations between field and DLA assessments of black spot indicating that

perhaps these two assessments are measuring different aspects of resistance. The narrow and broad sense heritability estimates from the combined analysis of field assessments is 0.3 and 0.4 respectively. An examination of the assessment data from the laboratory and the field showed that some seedlings were rated as resistant using both approaches.

Two microsatellite markers linked with *Rdr1* locus and one SCAR marker linked to *Rdr3* locus were found to be germplasm specific. The hybrid population 'Golden Gardens' x 'Homerun' that segregates for race 8 resistance was phenotyped for resistance to race 8 and genotyped for 38 SSR markers to assess if any of these SSR markers were associated with *Rdr3*. This resistance trait from the triploid source segregated non randomly and differentially in haploid and diploid gametes. None of the SSR markers examined were associated with *Rdr3*.

DEDICATION

This dissertation is dedicated to my grandma, Xiaoxian Zhang, who led me to the wonderful world of roses.

The work would never have been done without the unconditional support from my parents, Hong Fan and Qiusheng Dong, and my loving husband, Richard Geoffrey Charles Bowman.

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CHAPTER I

INTRODUCTION

Compared to the rose market 35 years ago, the production of garden roses has decreased 25 to 30% (Byrne et al., 2010), from 40 million roses down to 12 million field grown and 15-18 million pot grown rose bushes in 2012 (Hutton, 2012). This is thought to be because many rose cultivars have low tolerance to disease and abiotic stress. Thus roses among consumers appear to have the reputation of a high maintenance garden plant (Byrne et al., 2010). A survey conducted among both horticultural professionals and consumers by the Rose Hybridizer Association and Texas A&M University, indicated that disease resistance is the most important trait desired by the respondents. This was more important than fragrance, flower color, flower size and foliage characteristics (Waliczek et al., 2014). One of our goals is to develop disease resistant rose germplasm adapted to the hot and humid Texas climate (Byrne et al., 2007; Byrne, 2014).

Roses, which are distributed throughout the temperate regions of the Northern Hemisphere (Krussmann, 1981), have been important ornamental plants for more than five thousand years. There are thousands of cultivars for the garden, floriculture, medicinal, fragrance, and culinary industries (Gudin, 2000; Marriott and Austin, 2003; Shepherd, 1954). The rose industry contributes approximately a $400 million value from garden and landscape roses which is the major crop in the $2.81 billion wholesale US shrub market (AmericanHort, 2014).

The genus *Rosa* can be categorized into four subgenera, about 200 species and more than 20,000 commercial cultivars with a wide interspecific and intraspecific cross compatibility (Blechert and Debener, 2005; Cairns, 2000). Ploidy level in *Rosa* ranges from diploid to decaploid (Byrne and Crane, 2003; Jian et al., 2010), with most commercial cultivars being tetraploid, triploid or diploid hybrids derived from 8 to 10 wild diploid and a few tetraploid rose species (Byrne and Crane, 2003; Rajapakse et al., 2001; Ueckert et al., 2013; Zlesak, 2007; Zlesak, et al., 2010;).

As an ornamental crop, important traits in roses include fragrance, color, size, recurrent blooming, flower shape, double flower form, petal numbers, leaf color and form, neck form, prickles (stem and petiole), and growth habits (Byrne, 2013; Waliczek et al., 2014; Zlesak, 2007; Zlesak et al., 2013). Besides ornamental characters, disease resistance such as black spot disease resistance has become more important (Nybom, 2009). Genetic resistance would reduce the usage of agrochemicals and avoid environmental contamination and health related issues (Byrne, 2014; Debener and Byrne, 2014).

Black spot disease, the most important disease affecting garden roses globally, is caused by the water borne fungus *Diplocarpon rosae* Wolf (*Marssonina rosae* anamorph) (Nauta and Spooner, 2000). The typical symptoms of this disease include dark rounded spots with a feathery edge on the adaxial side of the leaves while the abaxial epidermis remain uninfected. The disease can cause the development of chlorosis around the lesion and eventually defoliation (Blechert and Debener, 2005; Gachomo et al., 2006; Horst and Cloyd, 2007). Eleven unique races of *D. rosae* have

been identified among the isolates obtained from North America and Europe (Whitaker et al., 2010).

Two types of disease resistance have been characterized in roses responding to black spot. Vertical or complete resistance which blocks sporulation and severely restricts the mycelial growth of the pathogen, is usually controlled by major genes (*Rdr*s) (Debener, 1998; von Malek and Debener, 1998; Whitaker et al., 2007; Yokoya et al., 2000). In rose the dominant resistance genes are pathogen race specific, indicating a gene-for-gene interaction pattern (von Malek and Debener, 1998).

Partial or horizontal resistance which appears to be non-race specific has also been identified in roses (Xue and Davidson, 1998). This resistance does not prevent infection of the pathogen, but rather delays disease development and results in reduced lesion size, reduced sporulation, and/or delayed infection after inoculation (Parlevliet, 1981; Whitaker and Hokanson, 2009; Xue and Davidson, 1998). Compared with complete resistance, partial resistance is more durable over the range of pathogenic races (Noack, 2003). The ideal disease resistant genotype should have both highly effective and long-lasting resistance to a broad spectrum of pathogenic races (Blechert and Debener, 2005), which can be achieved by pyramiding dominant complete resistance genes, obtaining strong partial resistance or by combining both types of resistances.

Black spot resistance is commonly evaluated in field trials at different geographic regions to expose the rose with a wider range of pathogenic races. These trials typically last 2-3 years to ensure sufficient disease pressure to properly assess the resistance of the plants (Carlson-Nilsson, 2000; Noack, 2003; Shupert, 2005). Lab based detached leaf

assay (DLA) is a tool for observing disease development efficiently under uniform and well controlled environmental conditions and inoculum levels (Hattendorf et al., 2004; von Malek and Debener, 1998; Whitaker and Hokanson, 2009a; b). Because single-conidial isolates are utilized in lab screening, the combination of compatible and incompatible interactions that are caused by various races in nature can be avoided (Blechert and Debener, 2005). However, disease measurement has to be done after only one cycle of disease development in DLA thus the differences among genotypes might not be as accentuated as compared to a field trial in which multiple cycles of pathogen development are common (Xue and Davidson, 1998). Other factors such as the physical status of the host plant, degradation of the leaves, missing observations on leaf abcission/defoliation in DLA and low or non-uniform inoculation levels in field assessment could all cause low correlation between these two methods of phenotyping (Johansson et al., 1992; Palmer et al., 1966; Zlesak et al., 2010).

Compared with phenotyping, which is time and labor consuming, molecular markers associated with specific traits is a highly desirable tool in plant breeding to identify candidate genotypes in designing crossing strategy, to negatively select against unwanted traits, and benefit selection by reducing the amount of seedlings that need to be phenotyped (Byrne, 2003; Debener and Byrne, 2014; Noack, 2003).

The objectives of this dissertation were to: 1) evaluate two methods of artificial inoculation for black spot disease evaluation and characterize rose genotypes for black spot resistance, 2) characterize the inheritance of partial disease resistance in a partial diallel mating design for diploid rose populations and 3) compare the estimation of

partial disease resistance conducted by field assessment and lab based detached leaf assay, 4) characterize the inheritance of complete disease resistance of race 8 controlled by *Rdr3* in a tetraploid x triploid population and molecular markers for their association with black spot resistance gene *Rdr3*.

CHAPTER II

DETACHED LEAF ASSAY AND WHOLE PLANT INOCULATION FOR MEASURING RESISTANCE TO *DIPLOCARPON ROSAE* IN *ROSA* SPP.

2.1 Synopsis

Black spot disease, caused by the fungus *Diplocarpon rosae* Wolf, is the most serious disease of garden roses (*Rosa spp*.) worldwide. Both complete resistance to specific races of the pathogen controlled by single dominant genes and partial non-race specific resistance controlled by multiple genes have been reported in rose. In this study, responses to *D. rosae* of 16 rose genotypes that were used as parents in hybrid populations were characterized with two inoculation methods: the detached leaf assay (DLA) and the whole plant inoculation (WPI) assay. The correlation between the two methods and their accuracy at measuring relative resistance among genotypes was analyzed. None of the roses assayed were completely resistant to black spot race 8. DLA is more sensitive than WPI in measuring relative resistance. However, the correlation coefficient between the methods is high (up to 0.58) which indicates that either can be utilized to characterize resistance against *D. rosae.* For hybrid populations generated from this germplasm, phenotyping data was collected by DLA.

2.2 Introduction

Roses (*Rosa* spp.) are one of the most important commercial flowers in the global market (Uggla and Carlson-Nilsson, 2005). For garden roses, the most serious disease is black spot, which has been reported globally (Horst and Cloyd, 2007; Nauta

and Spooner, 2000). The disease is caused by a water-borne hemibiotrophic fungus *Diplocarpon rosae* Wolf (*Marssonina rosae* anamorph) (Jenkins, 1955; Lyle and Massey, 1941; Luhmann et al., 2010; McClellan, 1953; Nauta and Spooner, 2000).

At the beginning of growing season, overwintering acervuli that formed subepidermally on the host plant release both one-celled spores (spermatia) and two-celled spores (conidia) to initiate the infection (Drewes-Alvarez, 2003; Horst and Cloyd, 2007; Nauta and Spooner, 2000). Secondary infections are started by conidia spread from the lesion area mainly by rain splash onto healthy tissue within the same plant or to adjacent plants (Drewes-Alvarez, 2003).

Typical symptoms of this disease include dark rounded spots with a feathery edge on the adaxial side of the leaves which generally develop chlorosis around the lesion, and causes the leaf to drop (Blechert and Debener, 2005; Gachomo et al., 2006; Horst and Cloyd, 2007). Through repeated infection cycles, the disease can reduce the growth, decrease the flower production or eventually cause the death of the plant (Carlson-Nilsson and Davidson, 2006; von Malek and Debener, 1998).

The optimal growth conditions of this pathogen are high humidity, frequent rains and a cool climate. The optimal temperatures are 18ºC and 24ºC for conidia germination and disease development, respectively (Drewes-Alvarez, 2003; Horst and Cloyd, 2007; Walker et al., 1995). When a compatible interaction occurs between the pathogen and host, the conidia will start to penetrate within 9-18 h with secondary mycelium developing on the second day, followed by the formation of haustorium within about 48 h. This is followed by fungal colonization, intracellular hyphae and the redirection of the

host nutrient metabolism (Blechert and Debener, 2005; Fernandez and Heath, 1990; Heath, 2002; Mendgen and Hahn, 2002; Voegele and Mendgen, 2003). Within 3-5 days, parallel and subcuticular hyphae strands are formed while visual symptoms can be detected in as little as 4 days (Whitaker et al., 2007). The fruiting body (acervuli) begins to form as early as 11 days and conidia are generally released 10-18 days after infection (Horst and Cloyd, 2007).

Different races of pathogen have been characterized by either the morphological traits (spore, hyphae, and the haustorium) or their interaction patterns with different rose genotypes (Aronescu, 1934; Drewes-Alvarez, 2003; Fries, 1815; Frick, 1943; Libert,1826). Multiple pathogenic races are found within one geographic region, and the distribution of pathogenic races of this fungus was uniform geographically in eastern North America (Whitaker and Hokanson, 2009) . This might be, in part, due to the national movement of rose plants and therefore the pathogen during their merchandising (Whitaker et al., 2007). While comparing isolates obtained from North America (USA and Canada) and Europe (France and Sweden), some geographic clustering was detected (Carlson-Nilsson, 2002). Recently, 15 isolates collected from North America and Europe were inoculated to a common set of rose cultivars which revealed 11 unique races of which some were found in two continents (Whitaker et al., 2010).

In the genus *Rosa*, the interaction with *D. rosae* can be categorized from resistant (incompatible with no acervuli development) to susceptible (compatible with acervuli development) (Blechert and Debener, 2005). Two types of resistance have been reported in roses. Vertical resistance, also referred to as complete resistance, conditions complete

resistance to the pathogen with no appearance of sporulation or mycelial growth. This is usually controlled by major genes (Debener, 1998; von Malek and Debener, 1998; Whitaker et al., 2007; Yokoya, 2000). Three dominant resistance genes *Rdr*s, have been identified responding to different genotypes of *D. rosae* (Hattendorf et al., 2004; von Malek and Debener, 1998; von Malek et al., 2000; Whitaker et al., 2010, Zlesak et al., 2010). In contrast, horizontal or partial resistance in roses is controlled by multiple genes with strong additive genetic effects and responds to different genotypes of *D. rosae* similarly (Zlesak et al., 2010). This type of resistance is referred to as partial resistance because the infection of the pathogen is not completely prevented but rather disease development is delayed resulting in reduced lesion size, reduced sporulation, and/or delayed infection after inoculation (Parlevliet, 1981; Whitaker and Hokanson, 2009; Xue and Davidson, 1998).

Black spot resistance on roses is commonly evaluated in field trials in different geographic regions to expose the germplasm to a wider range of pathogenic races. These trials usually last 2-3 years to ensure sufficient disease pressure to properly assess the black spot resistance of the rose genotypes (Carlson-Nilsson, 2000; Noack, 2003; Shupert, 2005). Lab based artificial inoculation such as the detached leaf assay (DLA) and whole plant inoculation (WPI) are the highly correlated tools for measuring disease development as they effectively control and optimize both environmental conditions and inoculum levels for consistent disease assessment (Hattendorf et al., 2004; von Malek and Debener, 1998; Whitaker and Hokanson, 2009a;b; Xue and Davidson, 1998). Because single-conidial isolates are utilized in lab screening, the combination of

compatible and incompatible interactions that are caused by various races in nature can be avoided (Blechert and Debener, 2005). However, disease measurements are done after only one cycle of disease development in artificial inoculation and the differences among genotypes might not be as accentuated as compared to a field trial in which multiple cycles of pathogen development are common (Xue and Davidson, 1998). Other factors such as the physical status of the host plant, degradation of the leaves, missing observations on leaf abcission/defoliation in DLA and low or non-uniform inoculation levels in field assessment could all lead to low correlations among these two methods of phenotyping (Johansson et al., 1992; Palmer et al., 1966; Zlesak et al., 2010).

To obtain a better understanding of the responses to the black spot fungus by the rose germplasm that is valuable to the breeding program, artificial inoculation is important for disease phenotyping. This study was conducted to characterize the disease resistance of 16 rose genotypes by DLA and WPI methods.

2.3 Materials and methods

2.3.1 Plant materials

Seven black spot susceptible roses ‘Cal Poly’, ‘Golden Gardens’, ‘Orange Honey’, ‘Red Fairy’, ‘Sweet Chariot’, ‘Vineyard Song’, and ‘Violette’, one with moderate resistance (‘Old Blush’), seven black spot resistant breeding lines 91/100-5, DD, FF, J06-20-14-3, J06-28-4-6, J06-30-3-6, M4-4, and one species rose *R. wichuriana* ‘Basye’s Thornless’ were used in this experiment (Byrne et al., 2010; Zlesak et al., 2010). All the resistant breeding lines have acquired their resistance from the resistant species *R. wichuriana* ‘Basye’s Thornless’ and/or the moderately resistant ‘Old Blush’.

The tetraploid line 91/100-5 is derived from *R. multiflora* in Germany (Debener, personal communication). Genotypes with different resistance abilities (Table 1) have the potential to be utilized as parents to create hybrid populations to characterize the inheritance of resistance.

All the plants were propagated from cuttings and were grown in one gallon pots containing a growth media of decomposed pine bark amended with Metro-Mix growing media® (Sun Gro Horticulture Canada CM Ltd, Agawam, WA) under the greenhouse environment for 3 month prior to the experiments. Nine individuals were randomly selected from each genotype for screening via artificial inoculation with three replications.

Table 1. Black spot resistance and ploidy level of rose germplasm.

Resistant	Susceptible
91/100-5 (4x)	Cal Poly (4x)
DD (2x)	Golden Gardens (4x)
FF (4x)	Orange Honey (4x)
J06-20-14-3 (2x)	Red Fairy (2x)
J06-28-4-6 (2x)	Sweet Chariot (2x)
J06-30-3-6 (2x)	Vineyard Song (2x)
M4-4 (2x)	Violette* (2x)
Old Blush (2x)	
R. wichuriana 'Basye's Thornless' (2x)	

* phenotype uncertain from field observation

2.3.2 Inoculation and data collection

Conidia of race 8 of *D. rosae*, which can be recognized by novel resistance gene *Rdr3*, were acquired from infected leaves of 'Cl. Pinkie' (Whitaker et al., 2010). The concentration of the conidia was adjusted to 1x 10^5 conidia/mL with the concentration measured by hemocytometer (W.W Grainger, Inc., Burr Ridge, IL). Inoculation was done by spraying the suspension of conidia onto the leaf tissue. This was left for 48 h and then the residual water was blotted off with a paper towel. The interactions between the host plants and pathogen were allowed to develop for DLA (14-16 days) and WPI (4 weeks) before categorizing the genotypes either as partially resistant to susceptible when spore-bearing acervuli are observed or completely resistant if no acervuli occur. In addition, the partial resistance among the susceptible plants was measured by the diameter of the largest individual lesion (lesion length) and the percentage of leaf area with symptoms (lesion size). The rating score of the leaf area with lesions was done as follows: 1 for 10%, 2 for 20%, 3 for 30%, 4 for 40% and 5 for 50% and above.

2.3.3 Detached leaf assay (DLA)

Up to seven young leaves from the 4^{th} to 6^{th} node from the apex of the shoot were collected from three plants of each rose genotype during each replication. After washing by DI water for 10 seconds on each side, the leaves were placed on wet paper towels in a transparent plastic container (152mm x 140mm x 59mm). The conidia suspension (1x 10^5 conidia/mL) was sprayed onto the leaves evenly with 0.75mL/spray. Forty-eight hours after inoculation, residual water was removed by blotting with a dry paper towel. DI water was added onto the paper towel without direct contact with the leaves to adjust

the humidity in the boxes to 100%. The inoculated leaves were then cultivated in the lab (~25ºC and 10 h photoperiod) for 14-16 days and then inspected for the incidence of acervuli under the dissecting microscope. The leaf area with symptoms (LAS) and lesion length (LL) data were collected. The entire experiment was repeated three times.

2.3.4 Whole plant inoculation (WPI)

Three Vigorously growing plants of each genotype were selected for WPI. Branches with a similar size were selected and sprayed with a conidia suspension (1x 10^5 conidia/mL) until the leaf surface was completely wet. A plastic bag was then used to cover the wet tissue for one week. Additional DI water was sprayed into the bags for high humidity (100%) maintenance. The inoculated plants were then maintained in the lab (~25ºC, 10 h photoperiod with a humidifier). Four weeks after inoculation, acervuli incidence was checked under the dissecting microscope. The relative black spot resistance was quantified by taking fata on the LAS, LL, and the number of inoculated leaves that abscised (NF). The entire experiment was repeated three times.

2.3.5 Statistical analysis

All statistical analysis was performed using SAS software, Version 9.3 SAS Institute Inc., Cary, NC, 1989-2010. The disease estimation was analyzed by ANOVA as a randomized complete block design. The testing of two inoculation methods was conducted under a standard environment and repeated three times, which was considered as block. The means of LL and LAS were compared by Fisher's LSD (Least Significant Difference) at P=0.05. Correlation coefficients of the components were generated from Pearson correlation analysis.

2.4 Results and discussion

Spore-bearing acervuli were observed on all genotypes whether using the DLA or WPI method, indicating that complete resistance to race 8 of *D. rosae* did not exist among the selected rose genotypes.

Using LL and LAS data, the most resistant genotypes as determined by field observations (*R. wichuriana* 'Basye's Thornless', M4-4, and J06-28-4-6) were clearly distinguishable from the roses rated as most susceptible to *D. rosae* ('Red Fairy', 'Sweet Chariot', 'Cal Poly', 'Vineyard Song' and 'Orange Honey') (Table 2). The best resolution among rose genotypes was with the LS data which was also able to separate other field resistant roses (91/100-5, DD, and J06-30-3-6) from the susceptible genotypes.

Table 2. Least square means of leaf area with symptoms (LAS) and black spot lesion length (LL) for 16 rose genotypes after infection with race 8 of *Diplocarpon rosae* with the detached leaf assay method.

	Least square means[z]	
Genotype	LAS	LL
91/100-5	1.50^{bcdef}	2.00^{abcde}
Cal Poly	1.98^{abcde}	2.33^{abcd}
DD	1.00^{f}	1.03^{cde}
FF	1.75^{abcde}	1.25^{cde}
Golden Gardens	2.08^{abcd}	2.50^{abc}
J06-20-14-3	1.28^{cdef}	1.15^{cde}
J06-28-4-6	1.08^{f}	0.49^{e}
J06-30-3-6	1.23^{def}	1.46^{cde}
M4-4	1.11^{ef}	0.86^{cde}
Old Blush	1.47^{cdef}	1.81^{bcde}
Orange Honey	2.46^{ab}	3.29^{ab}
R. wichuriana 'Basye's Thornless'	1.46^{cdef}	0.51^{de}
Red Fairy	2.53^{a}	3.44^{ab}
Sweet Chariot	2.49^{ab}	3.89^{a}
Vineyard Song	2.17^{abc}	2.50^{abc}
Violette	1.13^{def}	1.25^{cde}

[z] LSMeans within the components connected by the same letter are not significantly different at p = 0.05, with LSD adjustment.

Two traits, LAS and LL, which were used to characterize partial resistance, are positively correlated (R = 0.91 at P<0.0001) (Fig 1). Genotypes with a higher percentage of the leaf surface (LAS) being covered with lesions showed longer lesion length (LL), indicating either of these two traits could be used as indicator of the host plant response to the pathogen.

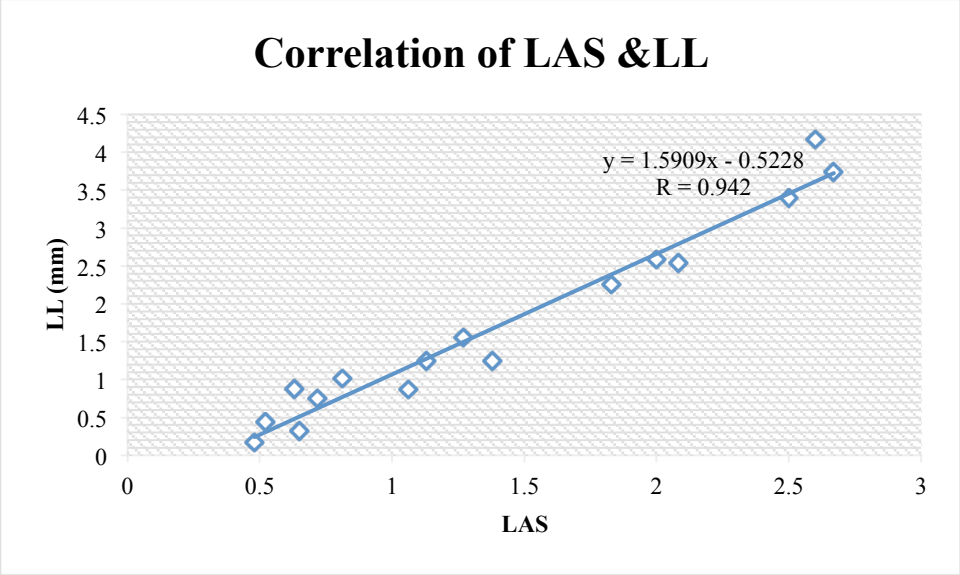


Fig. 1. Correlation of leaf area with symptoms (LAS) and lesion length (LL) measurements of partial resistance after infection with race 8 of *Diplocarpon rosae* with the detached leaf assay method.

When using the WPI to quantify the black spot resistance of the genotypes, it was found that the rose genotypes with higher resistance generally had lower LAS, LL, and NF when compared to the most susceptible rose genotypes but these groups were not consistently different (Table 3). This would suggest that the DLA approach is the better method for quantifying the relative partial resistance of rose to black spot. The

correlation among the various measures of black spot, both LAS and LL data from the WPI assay are well correlated to the LL and LAS data generated from the DLA protocol (R ranging from 0.46-0.58). LL and NF data from WPI are significantly correlated with R= 0.68. LAS and LL data from DLA are highly correlated with R=0.91 (Table 4).

Table 3. Least square means for number of fallen leaves (NF), leaf area with symptoms (LAS) and black spot lesion length (LL) for 16 rose genotypes after infection by race 8 of *Diplocarpon rosae* with the whole plant inoculation (WPI) method.

	Least square means[z]		
Genotype	NF	LAS	LL
91/100-5	0.00[b]	1.00[b]	2.00[abc]
Cal Poly	0.00[b]	1.11[b]	4.06[a]
DD	0.22[ab]	1.00[b]	1.46[bc]
FF	0.56[ab]	1.67[ab]	1.43[bc]
Golden Gardens	0.22[ab]	1.89[ab]	3.39[ab]
J06-20-14-3	0.56[ab]	1.56[ab]	1.56[bc]
J06-28-4-6	0.00[b]	1.00[b]	0.70[c]
J06-30-3-6	0.83[a]	1.75[ab]	1.50[bc]
M4-4	0.11[b]	1.56[ab]	2.11[abc]
Old Blush	0.22[ab]	1.67[ab]	2.83[abc]
Orange Honey	0.78[a]	2.44[a]	2.58[abc]
R. wichuriana Basye's Thornless	0.00[b]	1.17[b]	1.02[c]
Red Fairy	0.40[ab]	1.56[ab]	2.44[abc]
Sweet Chariot	0.56[ab]	1.89[ab]	2.28[abc]
Vineyard Song	0.78[a]	1.56[ab]	2.06[abc]
Violette	0.50[ab]	1.33[b]	2.81[abc]

[z] LSMeans within the components connected by the same letter are not significantly different at p = 0.05, with LSD adjustment for NF, LAS and LL.

Table 4. Correlation coefficients relating number of fallen leaves (NF), leaf area with symptoms (LAS), and black spot lesion length (LL) from whole plant inoculation (WPI) and leaf area with symptoms (LAS) and black spot lesion length (LL) from detached leaf assay (DLA).

		WPI			DLA	
		NF	LAS	LL	LAS	LL
WPI	LAS	0.68**				
	LL	-0.08	0.27			
DLA	LAS		0.58*	0.46		
	LL		0.58*	0.56*	0.91***	

*, **, ***Significant at P<0.05, 0.01 and 0.001 respectively (15 degrees of freedom).

From this study, several cultivars ('Red Fairy', 'Cal Poly', 'Sweet Chariot', 'Vineyard Song', and 'Orange Honey') were rated as very susceptible to black spot. Interestingly, the breeding line J06-30-3-6, which is derived from the wild species *R. wichurana* 'Basye's Thornless' and has an high level of partial resistance to black spot, had more leaves fallen under WPI than the other resistant lines (Table 3). 'Cal Poly' on the other hand, usually considered as a susceptible material based on field observation, showed no defoliation under WPI. It is possible that the different responses occurred after infection. Leaves fallen, although detrimental to the plant health, might reduce the secondary infection by decreasing the "reproductive supplement" of the pathogen while

‘Cal Poly’ provides the condition for the pathogen development by having the leaves attached.

2.5 Conclusions

The genotypes that were tested generally matched the responses to the pathogen in the field. DLA could distinguish the performance of the genotypes better than WPI and the two components of DLA were well correlated. As it is much easier to create a uniform humid environment under DLA as compared to WPI for a mass screening, DLA is more appropriate for the phenotyping of large populations and cultivar collections. Whitaker and Hokanson (2009b) also concluded that the detached leaf assay requires less input of time and facilities as compared to the whole plant assay. However, as LAS and LL data generated from WPI was correlated with LAS and LL data generated from DLA (Table 4), WPI could be utilized as a complementary characterization method to DLA for those genotypes whose leaves degraded easily.

CHAPTER III

GENETIC VARIANCES AND HERITABILITY OF BLACK SPOT PARTIAL RESISTANCE IN THE DIPLOID ROSE

3.1 Synopsis

Black spot disease, caused by the fungus *Diplocarpon rosae* Wolf, is the most serious disease of garden roses (*Rosa spp*.) worldwide. Dominant genes for complete resistance to specific races of the pathogen were identified in roses as *Rdr*s. Although partial resistance has also been studied, the genetic basis of this trait remains unidentified in our germplasm.

In this project, fifteen diploid populations were generated in 2010 and 2012 in a partial diallel mating design using 10 diploid parental genotypes including susceptible cultivars and resistant breeding lines. A detached leaf assay using race 8 of *D. rosae* was then conducted to assess partial resistance estimated by leaf area with symptoms (LAS) and lesion length (LL), respectively. Although the correlation of LAS and LL is significant, the correlation coefficient of these two components is 0.34, suggesting both components should be measured when evaluating disease development on progenies. The narrow sense heritability for partial resistance to black spot as estimated by both a genetic variances analysis and a mid-parent offspring regression ranged from 0.3-0.86.

The black spot resistance of the progeny of the population generated in 2010 were estimated by both field assessments in Texas during 2012-2013 and DLA. Field assessments were based on the percentage of the foliage with lesions. A 0 to 9 scale was

used to quantify black spot disease in the field. Field assessments conducted in fall were significantly (R = 0.1 - 0.2) although poorly correlated with DLA, while LAS and LL data collected from DLA also significantly correlated with R = 0.2. The normality of partial resistance data estimated in field assessment was better than in the data from DLA. A strong environmental effect was detected in the field trial indicating large variation among each evaluation. From the field assessment, narrow sense heritability of partial resistance estimated based on genetic variances ranged from 0.11-0.34 while broad sense heritability estimated as 0.4. Non-uniform or low inoculation level in the field results in unreliable assessments of black spot resistance in the first assessment (F12). With the increasing age of the trial the reliability of the black spot resistance assessments improves due to both increased inoculum levels and uniformity. .

3.2 Introduction

3.2.1 Domestication and breeding work

The commercial rose, which is one of the most popular ornamental plants, consists of thousands of cultivars for the garden, floriculture, medicinal, fragrance, and culinary industries (Marriott and Austin, 2003). This specialty crop generates approximately $400 million in revenue from the sales of bare root and containerized plants. The rose is an important component of the $2.81 billion US wholesale shrub market (AmericanHort, 2014).

The genus *Rosa* consists of four subgenera, about 200 species and more than 20,000 commercial cultivars with a wide interspecific and intraspecific cross compatibility (Blechert and Debener, 2005). Three out of four subgenera are monotypic:

Hulthemia (Dumort.) Focke, *Platyrhodon* (Hurst) Rehder, and *Hesperhodos* Cockerell (Nybom, 2009). The commercial rose has been developed mostly within the subgenera *Eurosa.* This subgenera includes 95% of all species and is subdivided into 10 sections: *Banksianae*, *Bracteatae*, *Indicae*, *Laevigatae*, *Synstylae*, *Gallicanae*, *Carolinae*, *Pimpinellifoliae*, *Caninae* and *Cinnamomeae* (Crespel and Mouchotte, 2003; Ritz et al., 2005).

The ploidy level in *Rosa* varies from diploid to decaploid, with greater ploidy diversification in regions with extreme environmental conditions (high altitude and latitude) (Byrne and Crane, 2003; Jian et al., 2010). Most commercial cultivars are tetraploid, triploid or diploid hybrids derived from 8 to 10 wild diploids and a few tetraploid rose species mostly from sections *Chinenses*, *Gallicanae* and *Synstylae* (Rajapakse et al., 2001; Ueckert et al., 2014; Zlesak et al., 2010).

Important traits in roses include fragrance, color, size, recurrent blooming, flower shape, flower form, petal numbers, leaf appearance, neck form, prickles (stem and petiole), and growth habits (Byrne, 2013; Waliczek et al., 2013; Zlesak, 2007; Zlesak et al., 2014). New trends such as garden roses that can produce cut flowers with petal color evolution, glossy foliage, attractive hips after fall defoliation and vigorous growth types not needing rootstock may be interesting for breeders as well (Chaanin, 2003; Gudin, 2003). Other than ornamental characters, disease resistance such as black spot disease resistance has become an important trait for consumers especially for garden roses due to the cost of the agrochemicals but also as a way to reduce the usage of agrochemicals and the environmental contamination and health related issues that are associated with their

use (Byrne, 2013; Debener and Byrne, 2014). Generally, if the commercial cultivars show adaptation to adverse environmental conditions (both biotic and abiotic stress), the ornamental feature could be appreciated for a longer time during the growing season, which increases their market demand (Nybom, 2009). Wild species and old cultivars distributed outside North America such as the highly disease-resistant old roses in China may provide genetic diversity for commercial cultivars (Guoliang, 2003).

Rosa is currently distributed in most temperate and subtropical regions globally. The rose was domesticated and first cultivated around 3000 BC in China and Egypt (Gudin, 2000; Nybom, 2009; Wissemann, 2003). North America, East Asia, and Europe/West Asia are major regions for the distribution of this genus. The breeding work of roses has been intensely conducted during the last two centuries during which more than 18,000 cultivars were registered and introduced to the market (Marriott and Austin, 2003). By hybridization of founder species of roses originated in Europe and China, many traits such as winter-hardiness, pest resistance, complex floral structure, bright petal color, and recurrent flowering were bred into modern roses (Crespel and Mouchotte, 2003). Based on the US patent record from 2010-2013, 10-20% of the roses registered in North America are miniatures or hybrid teas while 50-60% are either shrubs or floribunda roses (Byrne, 2014).

3.2.2 Genetic and mapping

Many wild rose species are diploid with a regular meiosis with 7 ring bivalents, but some exceptions do exist such as the species in sect. *Caninae* (DC.) Ser, also known as the dog roses, which has *canina* meiosis (Lim et al., 2005; Nybom, 2009). The

canina-meiosis is heterogamous, which means haploid pollen grains and tetraploid egg cells are formed during meiosis (Blackburn and Harrison 1921; Tackholm 1920, 1922). Therefore permanent pentaploid progeny will be generated with differential contribution of maternal (80%) and paternal genomes (20%) (Ritz and Wissemann, 2003; Wissemann and Hellwig, 1997).

In tetraploid roses, both allotetraploid (genomic combination with bivalents) and autotetraploids (genomic duplication with tetravalents) have been reported (Comai, 2005; Ramsey and Schemske, 2002). Both disomic and tetrasomic inheritance has been reported in the tetraploid rose although tetrasomic inheritance appears more frequent, (Gar et al., 2011; Koning-Boucoiran et al., 2012; Tsai, 2013). It is possible that due to the complex interspecific genomic background of roses, tetraploid roses have partially differentiated genomes which permits the concurrent existence of both disomic and tetrasomic inheritance (Ma et al., 1997; Ma et al., 2000).

A range of markers have been utilized for map construction including random amplified polymorphic DNA (RAPDs), amplified fragment-length polymorphisms (AFLPs), simple sequence repeats (SSRs), resistance gene analogues (RGAs), sequence characterized amplified regions (SCARs), and protein kinases (PKs) (Byrne, 2009). The mapping work on both diploid and tetraploid roses that has been conducted in several labs indicated 5-7 linkage groups. A consensus map of the rose genome was developed with SSR markers that were common over 4 diploid maps. This consensus map has 597 markers spread over 520 cM of chromosome length (Byrne, 2009; Spiller et al., 2010).

3.2.3 Challenges of breeding

Ploidy level among roses ranges from diploid to decaploid (x=7) with most modern roses being complex tetraploid, triploid and diploid hybrids (Debener and Linde, 2009; Jian et al., 2010; Rajapakse et al., 2001; Ueckert et al., 2013; Zhang et al., 2006; Zlesak, et al., 2010). Although interploidy crosses can be made in rose breeding and polyploid germplasm may be useful as a genetic bridge to create diverse genetic combinations, the dynamics of rose ploidy in interploidy crosses is poorly understood. Commonly these hybrids have reduced fertility which is a bottleneck for passing along target traits to the next generation (Byrne and Crane, 2003; Gudin, 2000; Leus, 2005; Ramsey and Schemske, 1998; Rowly, 1960; Zlesak, 2009). The fertility among roses vary dramatically even within the same ploidy level most probably due to the diverse interspecific genetic background of roses. In addition, some cultivars exhibit early dehiscence of the pollen (Spethmann and Feuerhahn, 2003).

The paucity of knowledge about rose genetics is a major challenge in rose breeding. Unlike the major annual agronomic and horticultural crops, the inheritance of only a few morphological and physiological traits of roses is known, which makes the breeding work less predictable when combining particular traits in one genotype (Crespel et al., 2002; Gudin, 2003; Zlesak, 2006).

Genetic work with rose populations is inhibited also due to low seed production per fruit and low germination rates which makes large cross populations hard to create (Crespel et al., 2002; Kaufmann et al., 2003). Nevertheless, commercial rose companies generally show excellent set and 40% or greater seed germination on selected breeding

parents indicating that this issue can be overcome (Byrne, personal communication). The fertility and germination issue in rose breeding can be optimized by more in depth understanding of rose sexual reproduction physiology such as in pollen post-harvest and seed physiology (Crespel and Mouchotte, 2003).

Despite the existence of those challenges, conventional hybridization is still the mainstream approach in rose breeding. Alternative technology including embryo rescue, protoplast fusion and ploidy level manipulation can be used as well to increase the genetic diversity possible by facilitating the survival of hybrids between distantly related rose genotypes (Crespel and Mouchotte, 2003). Marker assisted selection (MAS) is another approach that can benefit the breeding process by identifying specific phenotypic traits through molecular markers that are tightly linked to the genes that are controlling these traits (Byrne, 2003).

Among commercial rose cultivars, the diversity and frequency of disease resistant genes is limited. This makes the introgression of disease resistance genes from wild species a necessity (Debener, 2000). Some triploid roses produce viable haploid, diploid, and occasionally triploid gametes, which could be utilized as a bridge to introgress a target trait between diploid and tetraploid roses (Barden and Zlesak, 2004). The distribution of haploid, diploid and triploid male gametes produced by a triploid varies by the rose genotype. The percentage tetraploid progeny (diploid gametes) produced in a tetraploid x triploid cross ranged from 40% to 98% (Huylenbroeck, et al., 2005; Ueckert and Byrne, 2013; Zlesak et al., 2007).

3.2.4 Causal pathogen and symptom

Black spot disease is the most important disease that affects garden rose globally. The causal agent of this disease is a hemibiotrophic fungus *Diplocarpon rosae* Wolf (*Marssonina rosae* anamorph) which is an ascomycete that belongs to the *Dermateaceae* family (Nauta and Spooner, 2000). This pathogen is widely distributed throughout the world (Carlson-Nilsson and Davidson, 2006; Horst and Cloyd, 2007). Its host range is restricted to the *Rosa* genus although other species of *Diplocarpon* are pathogenic on other species in the *Rosaceae* family (Horst and Cloyd, 2007). The disease development is more severe under favorable environmental conditions or with a very compatible host-fungal interaction (Carlson-Nilsson and Davidson, 2006).

The asexual stage of this pathogen was first reported in 1815 in Sweden by Fries and referred as *Marssonina rosae* (Luhmann et al., 2010). The perfect stage of this disease (*Diplocarpon rosae*) was first reported by Wolf in 1912 on overwintered leaves in the USA (Drewes-Alvarez, 2003; Wolf, 1912). After that, the sexual stage of this pathogen has only been reported once in North America and twice in England, suggesting that the pathogen's ability to create genetic variation via the meiotic process may be limited (Horst and Cloyd, 2007; Walker et al., 1995).

Initial infection of a growing season is caused by both one-celled spores (spermatia) and two-celled spores (conidia) released from overwintering acervuli that formed subepidermally (Drewes-Alvarez, 2003; Horst and Cloyd, 2007; Nauta and Spooner, 2000). When successfully infected, a new disease cycle is initiated by

spreading conidia from the lesion area mainly by rain splash to healthy tissue within the same plant or to adjacent plants (Drewes-Alvarez, 2003).

The typical symptoms of this disease usually include dark rounded spots (up to 15 mm of diameter) with a feathery edge on the adaxial side of the leaves while the abaxial epidermis remains uninfected. Other symptoms common on susceptible genotypes are chlorosis around the lesion and after about 2 weeks, defoliation (Blechert and Debener, 2005; Gachomo et al., 2006; Horst and Cloyd, 2007). New shoots and leaves can regenerate after defoliation, but can be infected and/or abscised again. Thus the repeated infection cycles on infected plants can severely reduce the growth, decrease the flower production and/or eventually cause the death of the plant (von Malek and Debener, 1998). The level of winter damage could be increased as well because the new fall growth stimulated by defoliation may not have sufficient time to properly harden off before winter (Carlson-Nilsson and Davidson, 2006).

3.2.5 Genetic variability of D. rosae

Different races of the pathogen, which cause the differences in compatibility, are defined by their interaction patterns with different rose genotypes. The set of rose genotypes that can differentiate among pathogenic races of the fungus is called a differential set (Aronescu, 1934; Drewes- Alvarez, 2003; Fries, 1815; Frick, 1943; Libert,1826). Multiple pathogenic races have been reported in Germany (5), England (4), Canada (3), and Mississippi (7) (Debener, 1998; Svejda and Bolton, 1980; Spencer and Wood, 1992; Yokoya, 2000). When 15 isolates collected from North America and Europe were inoculated onto a common set of rose cultivars, only 11 unique races were

distinguished with some of the North American races being indistinguishable from some European races (Whitaker et al., 2010). Although within eastern North America, there was no geographic clustering, some was seen between continents (USA/Canada vs. France/Sweden) (Carlson-Nilsson, 2002). The lack of differentiation in race distribution in North America was suggested to be due to the movement of roses and thus, the pathogen, in commerce (Whitaker et al., 2007).

3.2.6 Black spot disease development

Under humid conditions, the conidia are usually moved by water splash and by insects (Walker et al., 1995). The optimal temperature for conidia germination is approximately 18ºC, and for disease development is 24ºC (Horst and Cloyd, 2007). For germination, the black spot conidia requires contact with water (Drewes-Alvarez, 2003). Therefore the morning dew or rainfall in the field creates a favorable condition for black spot infection of roses. In a greenhouse situation, black spot infections are minimal if there is good air circulation to reduce the humidity, watering is done to reduce splash, only pathogen free plants are brought into the greenhouse, and pathogen free water (DI water) is used.

If there is a compatible interaction between the pathogen and the host, the conidia will penetrate the cuticle and within about 48 h an haustorium will start to form. Successful establishment of an haustorium usually leads to fungal colonization of the leaf tissue via septate monokaryotic mycelium (Blechert and Debener, 2005). After forming an haustoria, the pathogen redirects the host nutrient metabolism, suppresses the host defenses, and increases of the host plant susceptibility to other pathogens

(Fernandez and Heath, 1990; Heath, 2002; Mendgen and Hahn, 2002; Voegele and Mendgen, 2003). During the necrotrophic stage, intracellular hyphae are often formed (Voegele and Mendgen, 2003).

In as little as 4 d after the spore germination, visual symptoms can be detected on susceptible hosts (Whitaker et al., 2007). Within 9-18 h conidia begin to germinate on moist leaves with the formation of secondary mycelium on the second day, and in 3-5 days parallel and subcuticular strands are formed. The fruiting body (acervuli) begin to form as early as 11 days and conidia may be released 10-18 days after infection (Horst and Cloyd, 2007).

3.2.7 Disease resistance: plant-pathogen interaction

In the genus *Rosa*, the interaction with *D. rosae* can be categorized from resistant (incompatible with no acervuli development) to susceptible (compatible with acervuli development). The susceptible or compatible interactions can be further divided into strongly to weakly susceptible based on the amount of asexual sporulation. Eight interaction types between the pathogen (race 6) and the host have been characterized (Blechert and Debener, 2005). In type 1, colonization of leaf tissue is facilitated by the growth of long distance subcuticular hyphae. The long-distance hyphae could bifurcate into short distance hyphae and grow intercellularly while forming intracellular haustoria. Later acervuli developed and conidia were released when the cuticle was ruptured by mature acervuli. In type 2, long straight subcuticular hyphae were detected with lateral poorly branched short-distance hyphae. Terminal haustoria were also formed as in type 1 but the hyphal network is less developed. Type 3 was defined by long-distance hyphae

with sparse and well-branched short-distance hyphae. In type 4 the colonization with subcuticular hyphae was weaker and the long-distance hyphae with parallel hyphae were shorter than in a type 1 interaction. In type 5, both long and short distance hyphae were poorly developed. In type 6 the fungus colonization was restricted to poorly branched short hyphal strands without long-distance hyphae but with few acervuli. However, cell-wall appositions were detected, as were necrotic spots at the point of infection. In type 7 after penetrating the cuticle, further fungus development was terminated with cell-wall appositions on one to three cells. Visible macroscopic necrosis was also detected. In type 8, neither necrotic spots nor fungal penetration of the cuticle were observed. In this resistant interaction, it was not clear whether the spores failed to germinate or germinated but failed to penetrate the cuticle.

D. rosae can successfully infect most rose cultivars with various degrees of severity although the number of resistant genotypes identified among wild roses appear to be greater than among cultivated genotypes. In a field trial conducted by Mynes et al. (The University of Tennessee, 2007) from 1995-2009 in Tennessee, 321 cultivars were evaluated for foliar leaf spot disease (black spot and cercospora) and defoliation at two locations for at least 3 years. Only 4% of the genotypes (13 cultivars) tested were defined as resistant. While in a single isolate inoculation test of 33 rose species, approximately 20% showed resistance including *R. caudata* (section Cinnamoneae), *R. gallica* (section Gallicanae), *R. wichurana*, *R. roxburghii*, *R. moyesii*, *R. multibracteata*, and *R. swegenzowii* var. *macrocarpa* revealed incompatible interactions with *D. rosae* (Blechert and Debener, 2005). Likewise, replicated field trial conducted by Texas A&M

University (Byrne et al., 2010) also confirmed that commercial cultivars with good resistance had rose species *Rosa wichurana*, *R. rugosa*, *R. multiflora*, *R. carolina*, *R. virginiana*, *R. laxa*, and *R. spinossisima* in their genetic background.

Vertical resistance which conditions complete resistance to the pathogen with no appearance of sporulation and mycelial growth is usually controlled by major genes (Debener, 1998; von Malek and Debener, 1998; Whitaker et al., 2007; Yokoya, 2000). The first discovered dominant black spot resistance gene is *Rdr1.* This race specific (race 3 and 6) resistance is derived from the diploid *R. multiflora* and is located on linkage group 1 of the rose genetic map (von Malek et al, 2000; von Malek and Debener, 1998; Whitaker et al., 2010, Zlesak et al., 2010). After identifying a single dominant resistance gene responding to a certain genotype of *D. rosae*, existence of a gene-for-gene interaction pattern was suggested between this pathogen and the host. However, further proof is still required with the identification of avirulence genes on *D. rosae* genome (von Malek & Debener, 1998). Later, another black spot disease resistance gene *Rdr2* was identified, which appears to be tightly linked to *Rdr1* (Debener et al. 1998; Hattendorf et al. 2004; Whitaker et al., 2010). Most recently, a novel resistance gene to race 8, *Rdr3,* was identified in a tetraploid population. This gene segregates independently of *Rdr1* (Whitaker et al., 2010).

Non-race specific partial resistance was also identified in roses (Xue and Davidson, 1998). This type of resistance does not prevent the infection of the pathogen, but rather delays disease development and results in reduced lesion size, reduced sporulation, and/or delayed infection after inoculation (Parlevliet, 1981; Whitaker and

Hokanson, 2009; Xue and Davidson, 1998). The polygenic control of this trait was later suggested by revealing a normal and continuous distribution of disease resistance in offspring families during field trial (Carlson-Nilsson, 2000; Korban et al., 1988; Shupert, 2005). Both diploid and tetraploid populations segregating for partial resistance ability showed strong additive genetic effects and significant general combining ability (Whitaker and Hokanson, 2009).

Cultivars that contain several vertical resistance genes may display strong resistance when released to the market, however, as it is planted in a wide region, it may become susceptible if it encounters a pathogenic race that can avoid the detection by the plants defensive response to the pathogen (Zlesak et al., 2010). The ideal disease resistant plant should have both highly effective and long-lasting resistance to a broad spectrum of pathogenic races (Blechert and Debener, 2005), which can be achieved by pyramiding several vertical or complete resistance genes, obtaining strong partial resistance or by combining both types of resistances.

3.2.8 Field and lab screening for disease resistance

Black spot resistance of germplasm is commonly evaluated in field trials and the seedlings with superior performance are selected and cloned for other field trials in different geographic regions to expose the rose to a greater number of pathogenic races (Noack, 2003).

Field assessment of rose disease resistance usually last 2-3 years to ensure that the plants are exposed to sufficiently high disease pressure to distinguish among levels of disease resistance (Carlson-Nilsson, 2000).

To speed up this process, it has been suggested that artificial inoculation can be done by adding infected plant tissue among the plants to be evaluated (Drewes-Alvarez, 1992). In practice, this is rarely done. More common is to plant new trials along with established trials, to plant susceptible genotypes throughout the trial, maintain susceptible plants in the trial until the trial is terminated, and plant at high density to encourage disease spread (Debener and Byrne, 2014).

Although more cycles of the pathogen development could occur for better assessment of disease resistance among genotypes in field trials, the establishment time for reliable evaluation is long (2-3 years) and thus costly (Carlson-Nilsson, 2000). Additionally, other fungus such as *Cercospora puderi* B.H. Davis which also causes leaf spotting may also attack roses in the field and interfere with the accuracy of the assessment (Horst and Cloyd, 2007). These pathogens may either weaken the host plant or cause error by being counted as black spot disease.

Lab based detached leaf assay is a tool for observing disease development which has better control of the humidity and inoculum levels, and is highly correlated with the whole plant inoculation method (von Malek and Debener, 1998; Hattendorf et al., 2004; Whitaker and Hokanson, 2009a;b). The advantage of lab screening using single-conidial isolates is that the combination of compatible and incompatible interactions on host plants, which is caused by various races in nature, can be avoided (Blechert and Debener, 2005). A disadvantage would be that a detached leaf assay conducted in lab usually allows only one cycle of disease development before the leaf tissue degrades. Therefore the differences among genotypes in lesion length and leaf area with symptoms

might not be as accentuated as compared to a field trial in which multiple cycles of pathogen development are common (Xue and Davidson, 1998). In addition, as the cuticle characteristics differ with leaf development and growth conditions (Johansson et al., 1992), so does the resistance of the leaf to the pathogen (Zlesak et al., 2010). Other concerns of conducting detached leaf assay that have been reported are problems with the degradation of the leaves and missing observations on leaf abcission/defoliation data (Palmer et al., 1966). Therefore experimental error or low correlation with field trial results (Zlesak et al., 2010) may derive from the stage of the leaves used in the detached leaf assay and/or their intrinsic ability not to degrade, different number of races, or low disease pressure in the field.

3.2.9 TAMU Rose Breeding and Genetics Program

The rose breeding program in Texas A&M University was initiated with the establishment of the Endowed Chair in Rose Genetics by Dr. Robert E. Basye in the early 1990s. Dr. Basye bred roses for over 50 years in Texas with the goal of producing roses well adapted to the climate of Texas that were "healthy rose bushes on which to hang those beautiful flowers" (Aggie Horticulture, 2014; Texas A&M Rose Breeding and Genetics Program, 2014).

The most famous Basye rose is "Belinda's Dream", which has a clear pink, fully double and strongly fragrant flower with a vigorous and disease resistant bush. Other cultivars bred by Dr. Basye are "Basye's Legacy" (1966), "Basye's Purple" (1968), "Basye's Myrrh Scented Rose" (1980), and "Basye's Blueberry" (1982) (Aggie

Horticulture, 2014). The wild rose cultivar *R. wichuriana* 'Basye's Thornless' used by Dr. Basye has been utilized as a primary source of partial resistance to black spot.

In 2007, Mr. Ralph Moore who was known as the "Father of the Miniature Rose" donated his rose cultivars and breeding material to the Rose Breeding and Genetics Program at Texas A&M University. His work, done in a dry zone of the central valley of California concentrated on developing unique floral traits in the rose. This germplasm complemented the disease resistant germplasm from the Basye and TAMU rose breeding program. Moore during his career, released several cultivars such as 'Gina's Rose' and 'My Stars' which are hybrids with Dr. Basye's materials (Texas A&M Rose Breeding and Genetics Program, 2014). Currently the TAMU Rose Breeding and Genetics program is combining the ornamental features from the rose developed by Moore with the good disease resistance from Basye and TAMU roses.

The TAMU Rose Breeding Program has used *R. wichuriana* 'Basye's Thornless' as a source of high black spot resistance and crossed it with several cultivars of *Rosa chinensis* ('Old Blush' and 'Ducher') to combine the high resistance from *R. wichuriana* with the flower characteristics and recurrent blooming trait of *R. chinensis*. After two to three generations of recombination and selection, recurrent blooming lines that are resistant to black spot disease (unpublished data) were identified. These lines have been crossed with several diploid commercial cultivars to generate populations with segregating phenotypes to develop good commercial lines with high resistance to black spot as well as for genetic studies.

3.2.10 Objectives

The goals of this study were to 1) characterize partial black spot disease resistance in diploid populations and examine the phenotypic distribution of ppopulations, components of genetic variances, and heritability of partial resistance; and 2) compare field assessment results with data obtained from DLA to assess the lab screening approach as a substitute method for field studies.

3.3 Materials and methods

3.3.1 Plant materials

Fifteen diploid populations were generated in a partial diallel mating design by crossing 5 black spot resistant lines (J06-20-14-3 (J14-3), J06-28-4-6 (J4-6), J06-30-3-3 (J3-3), J06-30-3-6 (J3-6), M4-4) and a moderately resistant line ('Old Blush') with 4 susceptible roses ('Little Chief' (LC), 'Red Fairy' (RF), 'Sweet Chariot' (SC), and 'Vineyard Song' (VS)) from 2010-2012 to create F_1 populations segregating for black spot resistance (Table 5). All the resistant lines have black spot resistance derived from *R. wichuriana* 'Basye's Thornless'. The moderately resistant and susceptible parents are commercial roses with excellent ornamental characteristics (Fig. 2).

	J14-3 (HR)	J4-6 (HR)	J3-6 (HR)	M4-4 (HR)	LC (S)	RF (S)	SC (S)	VS (S)
J14-3 (HR)					**X**	**X**	**X**	**X**
J4-6 (HR)						**X**		
J3-3 (HR)						**X**		
M4-4 (HR)							**X**	**X**
OB (MR)			**X**	**X**		**X**		
SC (S)	**X**	**X**		**X**				
VS (S)	**X**							

Fig. 2. Diploid rose progenies assayed for partial resistance to black spot. S = susceptible, MR = medium resistant, HR = high resistant, J06-20-14-3 = J14-3, J06-28-4-6 = J4-6, J06-30-3-3 = J3-3, J06-30-3-6 = J3-6, OB = 'Old Blush', LC = 'Little Chief', RF = 'Red Fairy', SC = 'Sweet Chariot', VS = 'Vineyard Song'. The female parents are listed vertically while the male parents are listed horizontally.

Table 5. Black spot resistance of the diploid parents of the populations. S = susceptible, MR = medium resistance, HR = high resistance, J06-20-14-3 = J14-3, J06-28-4-6 = J4-6, J06-30-3-3 = J3-3, J06-30-3-6 = J3-6, OB = 'Old Blush', LC = 'Little Chief', RF = 'Red Fairy', SC = 'Sweet Chariot', VS = 'Vineyard Song'.

Female	Male	Population size	Family name	Cross year
J14-3 (HR)	SC (S)	57	10074[b]	2010
			12080[a]	2012
SC (S)	J14-3 (HR)	58	12076[a]	2012
J14-3 (HR)	LC (S)	140	11061[a]	2011
			12046[a]	2012
J14-3 (HR)	RF (S)	130	12059[a]	2012
J14-3 (HR)	VS (S)	93	10073[b]	2010
VS (S)	J14-3 (HR)	12	10071[b]	2010
M4-4 (HR)	SC (S)	26	10075[b]	2010
			11118[a]	2011
SC (S)	M4-4 (HR)	118	10043[b]	2010
			12052[a]	2012
M4-4 (HR)	VS (S)	10	11112[a]	2011
J4-6 (HR)	RF (S)	97	10061[b]	2010
SC (S)	J4-6 (HR)	23	12044[a]	2012
OB (MR)	J3-6 (HR)	112	10038[b]	2010
OB (MR)	M4-4 (HR)	54	10041[b]	2010
OB (MR)	RF (S)	158	12062[a]	2012
J3-3 (HR)	RF (S)	38	10066[b]	2010
			12058[a]	2012

[a,b] The phenotypic data was collected in lab only and in both lab and field respectively

For populations generated in 2010 and 2011, a set of cuttings were collected from the field and propagated in November/October 2012 under mist in a peat and perlite mixture (Metro-Mix Professional Growing Mixes, Sun Gro Horticulture) in the greenhouse. The rooted plants were later transferred into 1-gallon pots in the same media

with slow release fertilizer (Osmocote 14-14-14, Scotts Miracle-Gro) and maintained in a greenhouse with a minimum day temperature of 20 ºC and a minimum night temperature of 15 ºC from January 2013- December 2014.

Populations that were generated in 2012 were germinated in the greenhouse then transferred into 1-gallon pots in June/July 2013 and maintained with the vegetatively propagated 2010 populations in the same greenhouse with the same growth media and fertilizer. At the age of 2 months, the plants were pruned back to synchronize shoot development to obtain shoots of similar physiological stage for inoculation. The same procedure was applied each time after collecting leaf samples.

3.3.2 Detached leaf assay (DLA)

From each individual, seven unfolded young leaves (4th-6th nodes from apical of each shoot) from 3 to 5 plants of each seedling for 2010 populations and from a single plant from each seedling for 2012 populations were collected for each inoculation. Conidia of race 8 of *D. rosae* was collected by washing the infected leaves of 'Cl. Pinkie'. The concentration of the conidia was adjusted to 1 x 10^5 conidia/mL. Each side of the leaf was washed with deionized (DI) water for 10 seconds and then placed onto a wet paper towel in a transparent plastic container (152 mm x 140 mm x 59 mm). Approximately 2 µL of the conidia suspension was evenly applied onto the leaves by spraying. After inoculation, the transparent plastic container was closed and the leaves and conidial broth were incubated for forty-eight hours. Residual water was then removed with a paper towel. The relative humidity in the boxes was maintained at 100% by adding supplemental DI water to the water towel. The incubation was continued in

the lab (~25ºC and 10 h photoperiod) for 14-16 days post inoculation (dpi). The entire experiment was repeated three times.

The partial (horizontal) resistance to the black spot fungus was assessed with two parameters. Disease development was quantified by the percentage of the leaf area with symptoms (LAS). LAS scores were categorized as 1%, 5%, 10%, 25%, 50%, or 75%. The rating scale was modified to be more refined (Xue and Davidson, 1998) as compared with the characterization on parental germplasm as in this experiment we were phenotyping populations with similar genetic background and not cultivars with diverse backgrounds. Lesion size was measured by the diameter (mm) of the largest individual lesion on the leaf surface (LL) (Fig. 3).

A.

B.

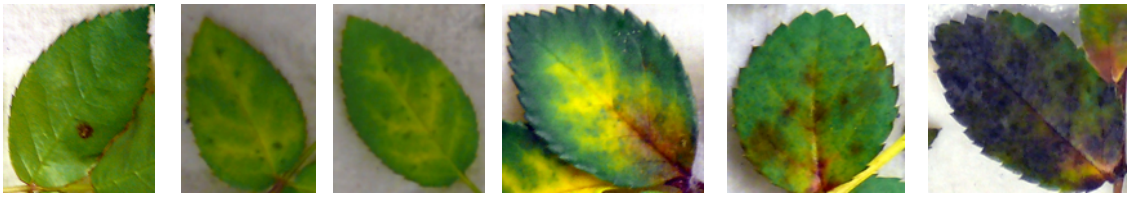


Fig. 3. (A) Spores bearing acervuli on infected leaf surface of 'Cl. Pinkie', (B) diagrammatic representation of leaf area with symptoms of black spot disease at 1%, 5%, 10%, 25%, 50%, or 75% in detached leaf assay.

3.3.3 Field assessment

In May 2012, the 1-year-old seedlings (Table 5.) were planted with double rows on the Horticulture Farm (1 m x 1 m x 3.5 m spacing) with weed barrier and drip irrigation at Texas A&M University at College Station. The irrigation was applied as needed without the application of fungicides or pesticides during the evaluation. Only maintenance treatment is pruning during March-April and August-September 2013 for removing dead tissue and restricting the plant size. The evaluation for black spot severity was done in the field in fall (October) 2012, spring (May) 2013, and fall (Oct-Nov) 2013 with temperature ranging from 18.7-29.6 °C and 8.5-18 °C for average high and low, respectively, and rainfall ranging from 55-231 mm (Table 6). (National Weather Service, 2014).

Table 6. Climatic records of College Station, TX for fall 2012 (October), spring 2013 (May), and fall 2013 (Oct-Nov) with average temperature (high and low) and rainfall.

Evaluation Time	**Temperature (°C)**		**Rainfall (mm)**
	Avg High	**Avg Low**	**Month/year**
October 2012	27.3	15.1	55/1046
May 2013	29.6	18	171/999
October-November 2013	27.1-18.7	15.8-8.5	231-116/999

Black spot severity was assessed based on the percentage of the foliage with lesions. A 0 to 9 scale was used with 0 = no lesions in the plant, 1 = occasional lesion on one or two leaves (1% of entire canopy), 2 = 20% infected canopy with any visible lesion, 3 = 30% infected canopy with any visible lesion, 4 = 40% infected canopy with any visible lesion, 5 = 50% infected canopy with any visible lesion, 6 = 60% infected canopy with any visible lesion, 7 = 70% infected canopy with any visible lesion, 8 = 80% infected canopy with any visible lesion, 9 = 90% and above infected canopy with any visible lesion. In 2013 November, an overall health rating was given by estimating the defoliation of the infected canopy (fallen leaves were estimated as the percentage of the canopy and counted as infected). A 0 to 9 scale was also used with 0 = no lesions and fallen leaves of the plant, 1 = occasional lesion on one or two leaves or fallen leaves (1% of entire canopy), 2 = 20% infected canopy with any visible lesion or reduced foliage, 3 = 30% infected canopy with any visible lesion or reduced foliage, 4 = 40% infected canopy with any visible lesion or reduced foliage, 5 = 50% infected canopy with any visible lesion or reduced foliage, 6 = 60% infected canopy with any visible lesion or reduced foliage, 7 = 70% infected canopy with any visible lesion or reduced foliage, 8 = 80% infected canopy with any visible lesion or reduced foliage, 9 = 90% and above infected canopy with any visible lesion or reduced foliage.

Disease assessment were done in October 2012 (F12), May 2013 (S13), November 2013 (F13Nov), October-November 2013 disease rating (F13BS), October-

November overall health rating 2013 (F13O), November 2013 evaluation (F13Nov), and October-November 2013 overall evaluation (F13).

3.3.4 Statistical analysis

Within each box, only the infected leaves were assessed with LAS and LL. The mean performance was calculated for each box and the single value was utilized in further analysis. The statistical analysis was conducted by using JMP software, Version 10, and SAS software 9.3, SAS Institute Inc., Cary, NC, 1989-2010. A square root transformation was done on the LAS and LL data to improve the data's normality in further analysis. The normality of the population data (original and transformed by taking square root) was analysed by Kolmogorov-Smirnov (K-S) test and skewness (SAS, 2012; Razali and Wah, 2011). Distribution of the population was estimated by both normal curve and kernel density curve for nonparametric distribution. Linear correlation of LL and LAS were estimated by Pearson correlation method.

From JMP®, genetic variances were calculated from restricted maximum likelihood (REML) method assuming all factors from this unbalanced design as random effects for more powerful estimation (Dieters et al. 1995; Littell, 1996). Variances of parents were considered as additive variance (V_A), progeny variance were considered as non-additive variance (V_D), repeated measurement variance was considered as variance of the environment (V_E), interaction of progeny and environment was also estimated as V_{GxE} (Connor et al., 2005). Narrow (h^2) and broad sense (H^2) heritability were estimated by the genetic variance from the ANOVA model, where $V_P=(V_A+V_D+V_{GxE}/E)$, $h^2 = V_A/V_P$, $H^2=(V_A+V_D)/V_P$ (Isik, 2009; Hallauer et al., 2010). Narrow-sense heritability was also

estimated by offspring mid-parent regression (Connor et al., 2005). Regression was generated by the average offspring (O) performance from reciprocal populations and the performance of the mid-parents (MP) which generate those populations, where h^2 = b =cov(O, MP)/cov(MP) (Falconer and Mackay, 1996), i.e. the slope of the regression is then the estimation of heritability with R^2 indicating the fitness of the regression.

3.4 Results

3.4.1 Density distribution of diploid populations

Based on the results of K-S normality test the LL and LAS data normality improved and skewness generally decreased after a square root transformation (Table 7). Thus all subsequent statistical analyses were done with the transformed data but it should be noted that the conclusions reached with the untransformed data and transformed data were not different.

Table 7. Normality (Kolmogorov-Smirnov) test on the distribution of raw and transformed (square root) data for partial resistance to race 8 of black spot that was measured by the leaf area with symptoms (LAS) and lesion length (LL) in detached leaf assays (DLA) for the diploid rose progenies. J06-20-14-3 = J14-3, J06-28-4-6 = J4-6, J06-30-3-3 = J3-3, J06-30-3-6 = J3-6, OB = 'Old Blush', LC = 'Little Chief', RF = 'Red Fairy', SC = 'Sweet Chariot', VS = 'Vineyard Song'.

	Kolmogorov-Smirnov				**Skewness**			
	LAS	**TranLAS**	**LL**	**TranLL**	**LAS**	**TranLAS**	**LL**	**TranLL**
J14-3xSC	**	*	NS	NS	1.29	0.44	0.61	0.28
SCxJ14-3	*	*	**	**	0.15	-0.57	0.95	0.76
J14-3xLC	**	**	**	NS	2.12	1.33	0.65	0.52
J14-3xRF	**	NS	**	NS	1.48	0.61	0.39	0.21
J14-3xVS	**	NS	NS	NS	0.94	0.38	0.42	0.16
VSxJ14-3	*	NS	NS	NS	0.78	0.83	-0.12	0.71
M4-4xSC	NS	NS	NS	NS	0.61	-0.18	0.15	0.00
SCxM4-4	**	*	**	*	0.62	-0.09	0.82	0.30
M4-4xVS	*	NS	NS	NS	1.83	1.34	1.28	1.17
J4-6xRF	**	NS	*	NS	0.95	0.39	0.74	0.32
SCxJ4-6	**	NS	NS	NS	0.73	-0.01	0.79	0.62
OBxJ3-6	*	**	*	NS	0.42	-0.05	0.37	0.15
OBxM4-4	NS	*	*	NS	1.03	0.24	0.50	0.09
OBxRF	**	*	**	**	1.30	0.49	-0.21	-0.43
J3-3xRF	*	NS	**	**	0.98	0.57	0.89	0.54

NS, *, **, ***Non-significant or significant at $p \leq 0.05$, 0.01, 0.005, respectively.

As with the DLA data, a square root transformation generally improved the normality and reduced the skewness of the field data.

The mean ratings for the S13 and F13 were higher than that of F12 reflecting a greater disease pressure in the later year and less escapes due to non-uniform pathogen distribution. This is further supported by the decreased skewness (0.85 in F12 to 0.12 in F13; less skewing towards resistance (Table 8)). Thus since there was little disease pressure in F12, subsequent analysis will focus on the rating data taken in S13 and F13. The distribution of disease rating of each the population becomes more normalized along with the repeated measurements from 2012 to 2013 (Table 9).

Table 8. Mean, range and normality (Kolmogorov-Smirnov) test on the black spot resistance ratings of 9 diploid rose populations from field assessment done in October 2012 (F12), May 2013 (S13), October-November 2013 overall evaluation (F13). Total seedling number is 386.

Field Assessment	Mean	Range	Kolmogorov-Smirnov	Skewness
F12	2.57	0-7.0	*	0.85
S13	4.12	1-9.0	*	0.64
F13	4.49	1-8.5	*	0.12

* significant at $p \leq 0.01$.

Table 9. Normality test of black spot disease resistance ability of progenies of 9 diploid populations conducted by Kolmogorov-Smirnov (K-S) test. Disease resistance was evaluated in the field (2012-2013) and in the laboratory with a detached leaf assay (DLA). Original data was transformed by taking square roots to improve its normality. J06-20-14-3 = J14-3, J06-28-4-6 = J4-6, J06-30-3-3 = J3-3, J06-30-3-6 = J3-6, OB = 'Old Blush', LC = 'Little Chief', RF = 'Red Fairy', SC = 'Sweet Chariot', VS = 'Vineyard Song'. Overall = field data combined from three seasons. Tran overall = transformed field data combined from three seasons. Tran LL = transformed LL. Tran LAS = transformed LAS.

	K-S									
	Over all	Tran Over all	F12	Tran F12	S13	Tran S13	F13	Tran F13	S13-F13	Tran S13-F13
J14-3 x SC	NS	NS	**	**	**	**	**	**	**	*
J14-3 x VS	NS	NS	**	**	**	**	*	**	**	**
J4-6 x RF	NS	NS	**	**	**	**	*	NS	**	**
M4-4 x SC	**	NS	NS	NS	NS	NS	NS	NS	*	NS
OB x J3-6	NS	NS	**	**	**	**	NS	NS	**	*
OB x M4-4	NS	NS	**	**	*	*	NS	NS	**	*
SC x M4-4	NS	NS	**	**	**	**	NS	NS	**	**
VS x J14-3	NS	NS	**	**	*	**	NS	NS	NS	NS
			Skewness							
J14-3 x SC			0.9	0.4	0.5	0.2	0.6	0.3	0.1	-0.2
J14-3 x VS			0.3	-0.7	0.5	0.1	0.1	-0.3	0.4	0.0
J4-6 x RF			0.8	0.1	0.4	0.1	0.5	0.0	0.4	0.0
M4-4 x SC			0.0	-0.4	0.0	-0.3	-0.3	-0.7	0.9	0.5
OB x J3-6			0.3	-1.2	0.5	0.1	0.2	-0.2	0.6	0.9
OB x M4-4			0.9	0.6	0.1	-0.2	-0.4	-1.0	0.4	0.0
SC x M4-4			1.5	0.5	0.3	-0.1	0.2	-0.1	0.4	0.0
VS x J14-3			1.3	1.3	2.0	1.3	-0.8	-1.1	0.2	-0.2

NS,*,**Not significant, significant at P<0.05 and 0.01, respectively.

3.4.2 Correlations among resistance assessments.

The correlation of individual progenies' partial resistance to black spot race 8 measured by LAS and LL (square root transformed data) from the detached leaf tests is 0.34 ($p < 0.0001$) (Fig. 4). The correlation of these two components was much higher ($R=0.9$) when estimating with resistant and susceptible parental materials, which have a wide range of responses to artificial inoculation with LAS ranging from 10%-42% and LL ranging from 0.1-7.14mm. This lower correlation of LAS and LL data possibly due to the resistance abilities of seedlings had smaller range in LL (ranging from 0.5-3.0 mm) while LAS remains similar (ranging from 1%-50%).

The two components from DLA, LAS and LL were not or only poorly correlated with field ratings (Table 10, Fig. 5). A similar correlation is seen between the two field evaluations (S13 vs F13 and other F13 evaluations) but the repeated evaluations within the F13 season were highly correlated indicating good consistency of the rating process.

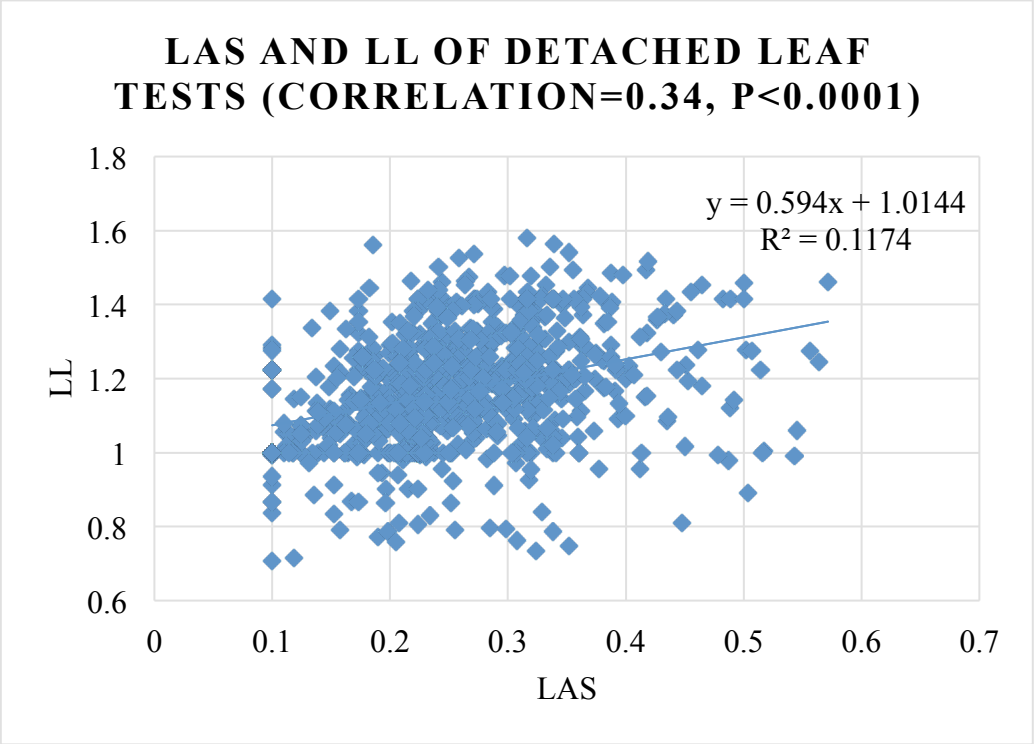


Fig. 4. Correlation of the individual seedlings of fifteen diploid rose populations of their partial resistance to black spot race 8 as measured by transformed (square root) data of lesion size (LAS) and length (LL) in detached leaf assays.

Table 10. Correlation coefficients relating field assessments analyzed by Pearson test. Disease assessment were done in the field in 2013 May (S13), 2013 November (F13Nov), 2013 October-November disease rating (F13BS), 2013 October-November overall health rating (F13O), 2013 November evaluation (F13Nov), 2013 October-November overall evaluation (F13) and in the laboratory using leaf area with symptoms (LAS), and black spot lesion length (LL) from detached leaf assay (DLA) inoculated with black spot fungus race 8. Data was transformed with a square root.

	S13	**F13BS**	**F13O**	**F13Nov**	**F13**	**LL**	**LAS**
S13		0.109	0.109	0.086	0.09	-0.042	0.019
		*	*	NS	NS	NS	NS
F13BS			0.708	0.915	0.948	0.091	-0.072
			***	***	***	NS	NS
F13O				0.887	0.878	0.117	-0.103
				***	***	*	*
F13Nov					0.979	0.121	-0.085
					***	*	NS
F13						0.106	-0.098
						*	NS

NS,*,**, ***Not significant, significant at P<0.05, 0.01 and 0.001 respectively.

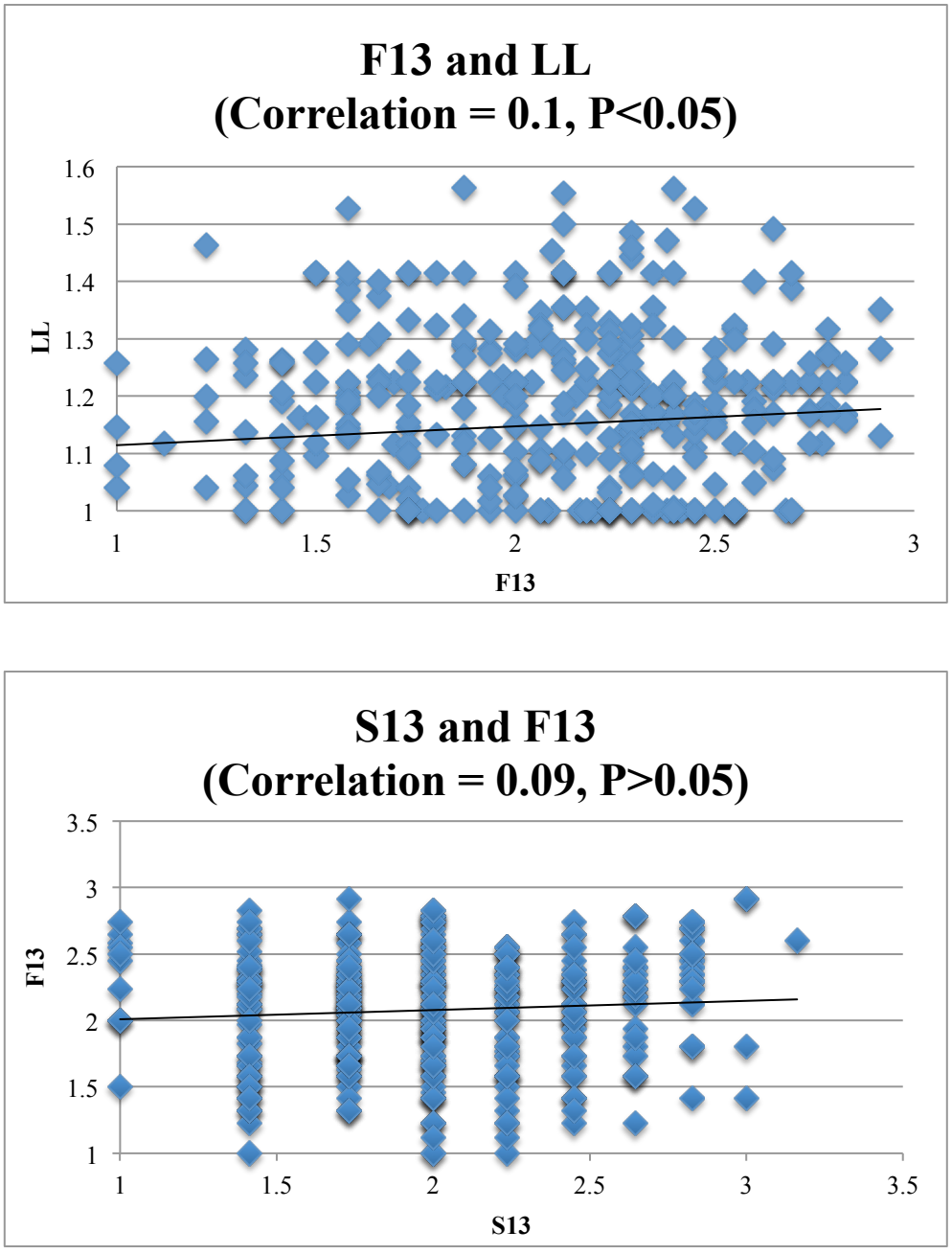


Fig. 5. Correlation of the individual seedlings of nine diploid rose populations of their partial resistance to black spot race 8 as measured by transformed (square root) data of length (LL) in detached leaf assays and field assessment in 2013 May (S13) and 2013 October-November overall evaluation (F13).

3.4.3 Genetic variation and estimation of heritability of disease assessments using the detached leaf assay

In this partial diallel mating design, narrow sense heritability (additive variance/phenotypic variance) was estimated being 0.3 and 0.4 for LAS and LL respectively, indicating this partial resistance trait is heritable from parents to progenies. The parental variance for LAS and LL account for 24% and 34% of the variance, while the progeny variance for LAS and LL accounts for 61% and 45% of total genetic variance respectively. Non-additive variances (0.006 and 0.016 for LAS and LL respectively) is greater than additive variance (0.002 and 0.012 for LAS and LL) in this incomplete diallel mating design indicating that progenies from specific cross combination could have better resistance ability than others (Table 11).

Table 11. Variances and estimated heritability of fifteen diploid rose populations measured by square root transformed leaf area with symptoms (LAS) and lesion length (LL) from detached leaf assay inoculated by race 8 of *Diplocarpon rosae*.

	Variances[x]			% of Total Variances[y]			
Components	σ^2_A	σ^2_D	σ^2_P	σ^2_A	σ^2_D	σ^2_P	Narrow sense heritability (h^2)[z]
LAS	0.002	0.006	0.009	23.9%	61.1%	85.0%	0.3
LL	0.012	0.016	0.028	33.9%	45.3%	79.2%	0.4

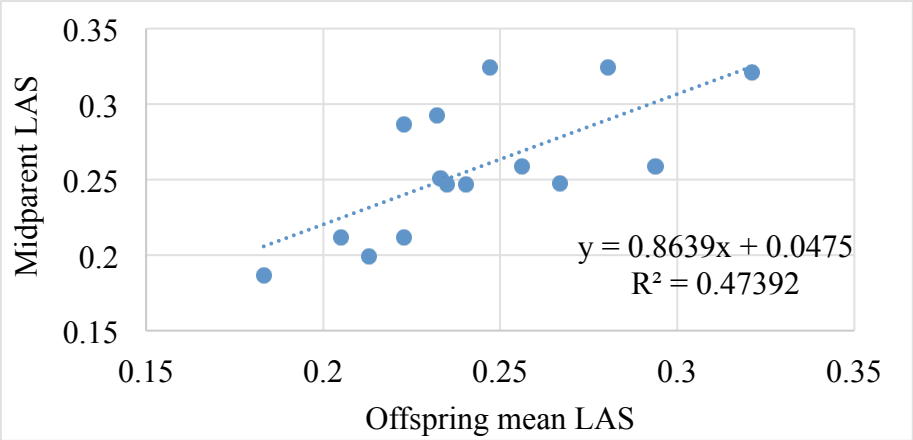
[x] σ^2_P = Phenotypic variances based on populations of individuals. σ^2_A = Additive variances based on variances of parents. σ^2_D = Non-additive variances based on variance of progeny.

[Y] % of Total Variances = percentage of total genetic variances caused by additive variances (σ^2_A), non-additive variances (σ^2_D), and phenotypic variances (σ^2_P).

[z] Narrow sense heritability = ratio of additive genetic variance to total phenotypic variance. $h^2 = \sigma^2_A / \sigma^2_P$.

Another approach utilized for estimating narrow sense heritability is the offspring mid-parent regression. The estimated narrow sense heritability of partial resistance measured by LAS and LL is 0.86 and 0.74 respectively. The fitness of the regressions (R^2) of LAS and LL was calculated as 0.47 and 0.43 respectively, indicating a fairly good estimation of the mid-parent and offspring performances (Fig. 6).

A.



B.

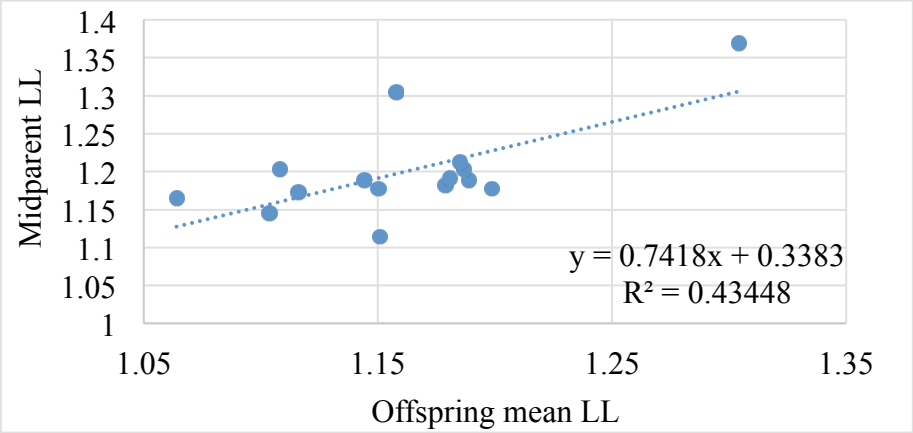


Fig. 6. The slope of mid-parent offspring regression estimates the narrow sense heritability of fifteen diploid populations measured by (A) leaf area with symptoms (LAS) and (B) lesion length (LL) from detached leaf assay inoculated by race 8 of *Diplocarpon rosae* with R^2 indicating the fitness of the regression. Original data was transformed by taking square-roots.

3.4.4 Phenotypes and heritability of partial black spot resistance estimated in the field

In the combined analysis of field assessments from second year, additive variances are higher (0.018) than non-additive variances (0.006) but both are very small compared to environmental variances (0.059). Because the interaction of genetic variances and environments is high (0.074), the narrow sense and broad sense heritability estimated from this model is 0.3 and 0.4 respectively indicating the partial disease resistance trait is moderately heritable.

Along with the repeated rating, both additive and non-variance are higher in S13 (0.0724 and 0.1432 respectively) than that in F13 (0.013 and 0.102 respectively). The narrow sense heritability estimated for each season is 0.34 and 0.11 for S13 and F13, respectively, lacking variance of genetic x environment (Table 12).

Table 12. Mean squares and genetic variances for black spot disease field ratings for 9 diploid populations for two seasons: May 2013 (S13), November 2013 (F13). Original data was transformed by taking square root. Additive variance (V_A), non-additive variance (V_D), environmental variance (V_E), Variance of genotypic interacts with environment (V_{GxE}), phenotypic variance (V_P), narrow (h^2) and broad (H^2) sense heritability. Female parent = F, male parent = M, progeny = P, environment = E. Heritability = ratio of genetic variance to total phenotypic variance. $h^2 = V_A/V_P$. $H^2 = V_D/V_P$.

	Mean Square		
Source	S13-F13	S13	F13
Female	0.016	0.033	0.0126
Male	0.002	0.0394	0.0002
Environment	0.059		
Progeny	0.006	0.1432	0.1018
Progeny x Environment	0.074		
Total	0.252	0.2156	0.1838
	Percentage of Total Variances		
Female	6.483	15.3	6.8
Male	0.734	18.3	0
Environment	23.317		
Progeny	2.496	66.4	55.4
Progeny x Environment	29.356		
	Genetic Variances		
V_A	0.018	0.0724	0.013
V_D	0.006	0.1432	0.102
V_P	0.062	0.2156	0.115
h^2	0.296	0.34	0.11
H^2	0.398		

3.5 Discussion and conclusion

3.5.1 Lab-based analysis

The square root transformation of the original LAS and LL data improves the normality and generally reduces the skewness of the distribution of the black spot assessments for diploid populations (Table 6). Normal distribution is important because it is the fundamental assumption of many statistical models including linear regression analysis and the analysis of variance (ANOVA) (Razali et al., 2011). The power of statistical analysis is improved with more normalized data.

More than half of the density distributions are normal for transformed LL (73%) data (P value <0.05 from K-S test), suggesting a proportional quantitative inheritance mode of this partial resistance trait (Table 6).

Genetic variances calculated from the mixed model (both LAS and LL) indicated that the additive variances explained 24%-34% of the total variances, which is lower than that of explained by non-additive variances (45%-61%) (Table 7). In contrast, the mid parent-progeny mean regression indicated that both measures were mainly additive in inheritance with heritability estimates of 0.74-0.86. Thus from a breeding point of view, the variance analysis would suggest that selection among families instead of within the families based on the high non-additive variance before selecting elite seedlings within progenies whereas the mid parent approach to estimating narrow sense heritability would suggest the best individual should be selected. From a complete factorial mating design of partial resistant and susceptible roses conducted by Whitaker and Hokanson (2009), within-family variances are much lower than that of between-

family variances. Therefore, selection for certain families (generated from certain parental combinations) followed by backcrossing to the parents with more advanced ornamental traits was suggested for future breeding (Table 7; Fig. 5).

Different results of narrow sense heritability estimated from genetic variances and offspring mid-parent regression might due to the structure of hybrid populations. This incomplete diallel mating design reduces the power of estimating genetic variances. In a factorial mating design conducted by Connor et al. (2005) with seven female and six male red raspberry, narrow sense heritability estimated by both methods were very similar for 3 traits and 2 years. For the offspring mid-parent regression, although 15 populations were used, most of the parent combinations are R x S, MR x S, MR x R, while S x S and R x R is lacking. When generating the regression, data points at lowest and highest region (bottom left and top right) are missing thus a higher estimation might be obtained if those combinations were included (Fig. 5). Since the diallel cross mating design is not complete and the variances of genetic x environment is lacking in the genetic variances estimation model, this offspring mid-parent regression might have higher power on estimating heritability.

3.5.2 Field assessment

Disease ratings among F12, S13, and F13 are not well correlated probably due to different inoculum levels during the evaluations. Mean disease ratings from the second fall (F13) revealed greater disease pressure and less skewing towards resistance which indicates less escapes and better inoculum distribution (Table 11, 12). Likewise, black spot evaluations conducted on *R. wichuriana* derived diploid populations by Shupert

(2005) also showed an improved ability to distinguish among levels of black spot resistance in the later evaluation (October) when the disease pressure (as indicated by mean black spot rating) was higher as compared to evaluations earlier in the year (May and July). Rose breeders and evaluators typically run the testing trials for 2–3 years to ensure sufficient inoculum in the field to be able to reliably assess the level of black spot resistance among the genotypes being tested (Byrne et al., 2010; Debener and Byrne, 2014).

The field and lab assessments of black spot were not well correlated (Table 9). These low to no correlations among the field and laboratory evaluations may be caused by several reasons.

1. The number of disease cycles possible differs in the field versus laboratory experiments. Multiple disease cycles occur during the field assessment within one growing season whereas the DLA only allows one disease cycle. In addition, in some genotypes the leaves begin to degrade before the test is over which decreases the confidence of the evaluations since it is difficult to distinguish between lesions caused by the black spot disease or another necrotrophic microbe infection.

2. During field assessment, some genotypes may have a large portion of canopy being infected but the size of the lesions were small (small LL) and covers only small percentage of the leaf area (low LAS). Therefore the same genotype might obtain a higher disease rating score than it was in the lab-based test when LL and LAS were used to estimate disease development.

3. More than one type of disease resistance mechanism may occur on the host plant. In lab test, only race 8 was utilized to estimate the partial resistance while other races in the field might trigger some dominant resistances or different degrees of restrictions of the disease. It is also possible that other mechanisms of horizontal/partial resistance operating in the field that was not measured in the lab.

4. Other diseases, such as cercospora leaf spot which is caused by *Cercospora rosicola* may cause confusion in field assessment since it has similar symptoms, (Whitaker and Hokanson, 2009). Cercospora has similar symptoms with black spot disease at an early stage of disease development (Horst, 1983). From the 2013 fall field assessment, 492 individuals in the field were infected with black spot disease only, 114 individuals were infected with cercospora only, and 221 individual were diagnosed with both diseases, in which 191 of them had black spot as primary disease and only 30 plants had cercospora as primary disease. These two diseases can be distinguished at later stage of development: black spot has feathery edges on the lesions while cercospora usually contains dead center on the lesion. Most of progenies have only a small portion of seedlings (less than 20%) primarily/only infected with cercospora except for J14-3 x VS (60%). Although cercospora may be the predominant pathogen on 30% of seedlings, infection with this disease might weaken the host resistance to black spot and make them more susceptible to black spot.

Therefore to improve the field assessment, several approaches can be recommended.

1. Place artificially infected plants between seedlings in the field during the growing season to increase disease stress intensity and randomize the inoculation source.

2. Evaluate during late fall during the rainy season in Texas on second/third year established field for stronger disease pressure, more developed plants which leads to an enhanced ability to distinguish between black spot and cercospora. Repeated measurements over one growing season may improve the evaluation for black spot damage and exclude the effects from cercospora leaf spot with more confidence (Mangandi et al., 2013). However, this would increase the labor input greatly.

3. More components can be included during the assessment such as rate of defoliation since older infected leaves may fall prior to the evaluation and thus not be counted as part of the infected canopy (Colbaugh et al., 2005), instead of just considering the percentage of foliage present with lesions.

Narrow sense heritability estimated from field assessment (0.3) is similar to that has been estimated from DLA (0.3-0.4) confirmed the partial disease resistance trait is moderate heritable. The additive variance estimated from field assessment is higher than non-additive variance when combining data obtained from two seasons of second year (Table 13). This result is in agreement with Whitaker and Hokanson (2009) in their complete mating design as well as with the work by Shupert (2005) who worked with black spot resistance from *R. wichuriana* 'Basye's Thornless' derived populations.

Each individual was ranked based on three criteria: the overall disease evaluation from the field in November 2013 (F13), LAS and LL from DLA. The selection was done separately for F13, LAS and LL data. The individuals which had the ranking score

within the selection index (top 30% of each population) of all three data were suggested for further evaluation. One to seven individuals from each of six populations were recommended for advanced selection. Of the 12 individuals selected from 394 seedlings for advanced evaluation regardless which population they are from, most belong to the populations J14-3 x VS (Table 13).

Table 13. Selection suggestions on black spot disease resistance of hybrid populations based on the performance ranking of field assessment in November evaluation 2013 (FII), leaf area with symptoms (LAS), and black spot lesion length (LL) from detached leaf assay (DLA) inoculated with black spot fungus race 8. J06-20-14-3 = J14-3, J06-28-4-6 = J4-6, J06-30-3-3 = J3-3, J06-30-3-6 = J3-6, OB = 'Old Blush', RF = 'Red Fairy', SC = 'Sweet Chariot', VS = 'Vineyard Song'.

		Combined Seedlings	
Cross	Selected Individual	Cross	Selected Individual
J14-3 x VS	10073-N007	J14-3 x VS	10073-N007
	10073-N029		10073-N029
	10073-N039		10073-N039
	10073-N106		10073-NoLabel2
	10073-NoLabel2		10073-NoLabel3
	10073-NoLabel3		10073-NoLabel4
	10073-NoLabel4	J4-6 x RF	10061-N046
J4-6 x RF	10061-N046		10061-N112
	10061-N076	M4-4 x SC	10074-N078
	10061-N077	OB x J3-6	10038-N026
	10061-N112	OB x M4-4	10041-N002
	10074-N007		10041-N049
M4-4 x SC	10074-N033		
	10074-N069		
	10074-N078		
	10038-N026		
OB x J3-6	10038-N055		
	10038-N099		
	10038-N129		
OB x M4-4	10041-N025		
SC x M4-4	10043-N034		
	10043-N049		

CHAPTER IV

MOLECULAR MARKER ASSISTED SELECTION IN DISEASE RESISTANCE ROSE BREEDING

4.1 Synopsis

Black spot disease, caused by fungus *Diplocarpon rosae* Wolf, is the most serious disease of roses (*Rosa spp*.) worldwide in the outdoor landscape. Dominant genes for complete resistance were identified in roses as *Rdr*s. From a breeding perspective, a rapid screening of breeding materials by molecular markers is beneficial for identifying the resistant germplasm. To characterize molecular markers in a broad spectrum of rose germplasm, two microsatellite markers (155 at 0 cM and 69E24 at 0.1 cM distance) linked to *Rdr1* (resistance to race 3) were used to screen 208 rose genotypes. In addition one SCAR marker (ND5E) (9.1 cM distance) linked to *Rdr3* (resistance to race 8) was used to screen 56 rose genotypes. Twenty-five of these genotypes have known phenotypes for black spot resistance to race 8.

The SSR markers associated with *Rdr1* detected 75%-100% of the resistant genotypes, however, the false positive rate was also high (42%-50%). Therefore, the markers appear to be germplasm specific as in the populations derived from the original source of resistance, the linkage is excellent. The detection rate of the SCAR marker ND5E, which is associated with *Rdr3,* is relatively low (60%), though false positive rate is very low (5%). Thus the presence of the ND5E marker as a marker for *Rdr3* gene is not reliable in a wide range of rose germplasm either.

The hybrid population ‘Golden Gardens’ x ‘Homerun’ that segregates for *Rdr3* which conditions race 8 resistance were phenotyped and assessed for associations with a set of SSR markers *Rdr3*. This resistance trait from the triploid source segregated non randomly and differentially in haploid and diploid gametes. None of the SSR markers examined were associated with *Rdr3*.

4.2 Introduction

4.2.1 Rose breeding

Rose as a globally important ornamental plant is phenotypically diverse and highly heterozygous (Debener and Linde 2009; Dugo et al. 2005; Hibrand-Saint Oyant et al. 2008). It has been broadly utilized as garden and landscape plants, potted plants, cut flowers, and a source of aromatic oil and vitamin C (rose hips) (Gudin, 2000; Wen et al., 2006). Of the approximately 200 species in *Rosa* genus which range from diploid to decaploid (x = 7), only 8-10 diploid species and a few tetraploid species contributed to the genetic background of the more than 20,000 modern cultivars in existence (Gudin, 2000). Most modern roses are complex tetraploid, triploid and diploid hybrids (Debener and Linde, 2009; Rajapakse et al., Ueckert et al., 2013; Zhang et al., 2006; 2001; Zlesak, et al., 2010).

The genetic study of roses is relatively new endeavor as compared the domestication and breeding of the rose. The inheritance of only a few important morphological and physiological traits are reported (Crespel et al. 2002; Debener et al. 2001; Gudin, 2000; Hibrand-Saint Oyant et al. 2008). The genetic research of roses is difficult for several reasons: high heterozygosity of the cultivars (Berninger, 1992; Gudin

and Mouchotte, 1996; Rowley, 1966), various ploidy levels (Berninger, 1992; Jacob et al., 1996) and frequent poor fertility resulting in small populations that can be studied (Buck, 1960; Gudin, 1995; Gudin and Mouchotte, 1996). Due to the high heterozygosity of *Rosa* genus, the pseudo-test-cross strategy is used to develop genetic maps from segregating populations (Crespel et al., 2002; Debener et al., 1999; Dugo et al., 2005; Gar, 2011; Grattapaglia and Sederoff, 1994; Hossein et al., 2012; Hibrand-Saint Oyant, et al., Koning-Coucoiran, et al., 2012; Moghaddam et al., 2010; Rajapakse, et al., 2001; Spiller et al., 2011; Yan et al., 2005; Zhang et al., 2006; 2007).

Rose chromosomes are considered relatively small. In diploid roses, 2C DNA size varies from 0.83 to 1.30 pg (Roberts et al., 2009). The rose genome size is about 600 Mb (Rajapakse et al., 2001; Yokoya et al., 2000), which is about four times larger than that of the model crop *Arabidopsis thaliana* (L.) Heynh (Zhang et al., 2006). Due to the low chromosome number and small genome size, the rose has the potential of being a model system along with *Prunus* and *Malus* for the *Rosaceae* family (Biber et al. 2010; Debener and Linde 2009; Whitaker et al. 2010; Zhang et al. 2006).

The breeding goals in roses have always been the introgression of alleles of interest from wild or exotic materials into elite breeding lines. Major trends in garden rose breeding are the development of low-maintenance (disease resistance, winter hardiness, shade tolerance) shrubs with compact growth types and free-blooming habits (Byrne, 2013; Zlesak, 2007).

4.2.2 Black spot disease of roses

For the *Rosa* genus, black spot disease is the most important disease affecting the garden rose globally. The causal agent of this disease is a hemibiotrophic fungus *Diplocarpon rosae* Wolf (*Marssonina rosae* anamorph) (Nauta and Spooner, 2000). This disease on rose usually causes dark rounded spots with a feathery edge on the adaxial side of the leaves while the abaxial epidermis remains green and uninfected. Other common symptoms on susceptible genotypes is chlorosis around the lesion and about 2 weeks later defoliation may occur in severe cases (Blechert and Debener, 2005; Horst, 1983). New shoots and leaves regenerated after defoliation may also become infected and/or abscise again. Consequently this repeated infection cycle can severely reduce growth, decrease flower production and eventually kill the plant (von Malek and Debener, 1998).

The initial infection for the growing season is caused by spores released via rain splash from fallen leaves from the previous year or from fruiting body structure (acervuli) formed on stems and leaves (Horst and Cloyd, 2007; Nauta and Spooner, 2000). Although both one-celled spores (spermatia) and two-celled conidia can be released from acervuli, these structures release predominantly two celled conidia, which are capable of overwintering when formed subepidermally (Drewes-Alvarez, 2003). If the interaction between the pathogen and host is compatible, the conidia will penetrate the cuticle and within about 48 h, an haustoria will start to form (Blechert and Debener, 2005). In as little as 4 d after the spore germination, visual symptoms can be detected on susceptible hosts under humid conditions (Walker et al., 1995; Whitaker et al., 2007).

Within 5 days, reproductive spore conidia begin to develop and after 7 days the acervuli disrupts the leaf epidermal surface and the conidiospores are released. These are spread by water splash (rain or irrigation) and infect other healthy tissue (Horst and Cloyd, 2007). Either black or brownish spots with irregular edges will appear on the adaxial side of the leaves while the abaxial epidermis remain unaffected. Approximately two weeks post inoculation, defoliation can be observed on susceptible rose genotypes (Blechert and Debener, 2005).

Different races of the pathogen, which cause the differences in compatibility, are defined by their interaction patterns with different rose genotypes. The set of rose genotypes that can differentiate among pathogenic races of the fungus is called a differential set (Aronescu, 1934; Drewes- Alvarez, 2003; Fries, 1815; Frick, 1943; Libert,1826). Multiple pathogenic races have been reported in Germany (5), England (4), Canada (3), and Mississippi (7) (Debener, 1998; Spencer and Wood, 1992; Svejda and Bolton, 1980; Yokoya, 2000). When 15 isolates collected from North America and Europe were inoculated onto a common set of rose cultivars, only 11 unique races were distinguished with some of the North American races being indistinguishable from some European races (Whitaker et al., 2010). Although within eastern North America, there was no geographic clustering, some was seen between continents (USA/Canada vs. France/Sweden) (Carlson-Nilsson, 2002). The lack of differentiation in race distribution in North America was suggested to be due to the movement of roses and thus, the pathogen, in commerce (Whitaker et al., 2007).

In roses, the interaction types can be categorized from resistant to susceptible, and the compatible interactions can be further divided into strongly and weakly susceptible based on the amount of asexual sporulation (Blechert and Debener, 2005). Vertical resistance, which is usually controlled by major genes, conditions complete resistance to the pathogen and prevents sporulation and mycelial growth (Debener, 1998; von Malek and Debener, 1998; Whitaker et al., 2007; Yokoya, 2000). The first such gene, Rdr1, was identified in the diploid *Rosa multiflora*. The dominant gene *Rdr1*, located on linkage group 1 of the rose genetic map, conditions resistance to race 3 and 6 (von Malek et al, 2000; von Malek and Debener, 1998; Whitaker et al., 2010; Zlesak et al., 2010). A gene-for-gene interaction pattern therefore was suggested between this pathogen and the host. However, a further proof with the identification of avirulence genes on *D. rosae* genome is required to support this hypothesis (von Malek and Debener, 1998). From similar *R. multiflora* derived diploid populations, another black spot disease resistance gene *Rdr2* was identified by inoculation with race 4. *Rdr2* appears to be linked within 10 cM of *Rdr1* (Debener et al. 1998; Debener and Linde, 2009; Hattendorf et al. 2004; Kaufmann et al., 2003; Malek and Debener, 1998; Whitaker et al., 2010; Yan et al., 2005; Zhang, 2003;). Most recently, a novel resistance gene to race 8, *Rdr3,* was identified in a tetraploid population. This gene segregates independently of *Rdr1* (Whitaker et al., 2010; Zlesak et al., 2010).

Non-race specific partial or horizontal resistance was also characterized on various rose cultivars using a range of fungal growth components (Xue and Davidson, 1998). Although this type of resistance does not prevent infection of the pathogen, it

delays disease development by affecting fungal growth and reproduction which results in reduced lesion size, reduced sporulation, and/or delayed infection after inoculation (Parlevliet, 1981; Whitaker and Hokanson, 2009; Xue and Davidson, 1998). The polygenic control of this trait was later suggested based on a normal and continuous distribution of disease resistance in progenies from field trials (Carlson-Nilsson, 2000; Korban et al., 1988; Shupert, 2005). Both diploid and tetraploid genotypes with partial resistance showed strong additive genetic effects and significant general combining ability (Whitaker and Hokanson, 2009). Thus this type of resistance appears to be controlled by multiple genes (QTLs). Furthermore, it is thought to provide more durable resistance especially when a novel pathogen genotype exists in the population (McDonald and Linde, 2002).

Black spot resistance of germplasm is commonly evaluated in field trials for approximately 2-3 years to ensure sufficient disease pressure and plant mass for effective disease assessment (Carlson-Nilsson, 2000; Lühmann et al., 2010; Noack, 2003; Saunders, 1970; Shupert, 2005). Approaches suggested to increase the disease pressure in field evaluation trials include moving infected plant tissue among the plants, planting new trials along with established trials with high inoculum levels, and maintaining susceptible genotypes throughout the field (Debener and Byrne, 2014; Drewes-Alvarez, 1992).

Although field trials would allow more cycles of pathogen to develop, the long assessment time (2-3 years) generates a high cost (Carlson-Nilsson, 2000). Other fungal diseases on roses such as *Cercospora rosicola*, which also cause a rose leaf spot (Horst

and Cloyd, 2007), could interfere with the evaluation as well. The reduced accuracy of disease assessment may come from similar symptoms of different diseases or weakening health of the host plant by infection from other pathogens.

An alternative method to evaluate disease development is the lab based detached leaf assay (DLA) using single-conidial isolates, which could allow more efficient disease development due to better control of the humidity and inoculum levels (Hattendorf et al., 2004; von Malek and Debener, 1998; Whitaker and Hokanson, 2009a;b). Inoculation with a single isolate can avoid the combination of compatible and incompatible interactions on host plants from multiple races, which may occur in a field trial (Blechert and Debener, 2005). However, DLA only allows one cycle of disease development before the leaf tissue degrades. This may limit the ability to distinguish among genotypes as compared to a field test where the plants experience the accumulated effects of multiple disease cycles differences among genotypes might not be distinguished by measuring components such as lesion length (LL) and leaf area with symptoms (LAS) (Horst and Cloyd, 2007; Xue and Davidson, 1998).

Three disadvantages of the DLA have been reported. 1.) DLA uses young leaves to measure resistance whereas in the field, frequently the first infections of black spot appear on the lower, more mature leaves (Johansson et al., 1992; Zlesak et al., 2010). 2.) Degradation of the leaves varies with the genotype and may affect the rose's resistance to the disease and the ability to measure lesion development. 3. DLA does not permit observations on leaf abcission which is a common symptom in the field (Palmer et al.,

1966). Thus there may not be a strong correlation between DLA and field results at some measuring components (Zlesak et al., 2010).

4.2.3 Molecular markers in rose genetics and mapping

Although rose is an economically important crop, knowledge of rose genetics, genome structure, and the function of rose genes are still limited. This lack of information could be improved by the development of molecular and biotechnological tools (Debener and Linde, 2009). In rose breeding, molecular markers associated with target traits could be utilized to identify candidate genotypes for designing crosses to optimize the probability of best gene combinations, select candidate individuals to reduce the amount of seedlings for phenotyping, and/or negatively select against unwanted traits during introgression (Byrne, 2003; Noack, 2003; Debener and Byrne, 2014; Hibrand-Saint Oyant et al., 2008; Hosseini Moghaddam et al., 2012; Spiller et al., 2011; Yan et al., 2005).

Various types of molecular markers have been utilized in rose genetics including amplified fragment-length polymorphisms (AFLP), randomly amplified polymorphic DNA (RAPD), simple sequence repeat (SSR) markers, protein kinase specific fragments (PK) and resistance gene analogues (RGA) markers (Hosseini Moghaddam et al., 2012). Microsatellites or SSR are short DNA motifs of 1-6 bp, which distributed in clusters of 50 to 100 bp. SSR markers are relatively abundant, usually highly polymorphic and robust in a PCR-based approach. Since SSRs can be co-dominant, it is useful when characterizing multiple alleles in the construction of polyploid maps. Therefore they have been broadly utilized in genetic linkage maps and germplasm characterization

(Debener et al., 1996; Mohapatra and Rout, 2006; Spiller et al., 2010; Zhang et al., 2006).

In roses, the mapping strategy that has been utilized is “double pseudo test cross strategy”, in which independent maps are constructed for each parent followed by joining the linkage groups with common markers (Debener and Linde, 2009). Linkage maps were constructed on both diploid (Crespel et al. 2002; Debener and Mattiesch 1999; Dugo et al. 2005; Linde et al. 2006; Yan et al. 2005) and tetraploid (Gar et al. 2011; Koning-Boucoiran et al., 2012; Rajapakse et al., 2001) roses and aligned and integrated by SSR markers (Ballard et al., 1996; Hibrand-Saint Oyant et al., 2008; Spiller et al., 2010; Tsai, 2014; Zhang, 2003; Zhang et al., 2006).

Linkage maps could be utilized to locate monogenic traits and quantitative traits controlled by multiple genes (Collard et al. 2005). Several important traits have been placed on rose maps including flower color (Debener and Mattiesch, 1999), petal number and double corolla (Crespel et al., 2002; Debener et al., 2001; Hibrand-Saint Oyant et al., 2008), prickles (Crespel et al., 2002; Linde et al., 2006; Rajapakse et al., 2001), flowering time (Dugo et al., 2005; Hibrand-Saint Oyant et al., 2008; Kawamura et al., 2011), leaf size (Dugo et al., 2005; Yan et al., 2005), number of internodes, total dry weight (Yan et al., 2005), inflorescence architecture (Kawamura et al., 2011), powdery mildew resistance (Dugo et al., 2005; Linde et al., 2006), and black spot resistance (Debener and Mattiesch, 1999). For black spot disease resistance, both major gene controlled complete resistance (Debener, 1998; Hattendorf et al. 2004; von Malek and Debener, 1998; von Malek et al, 2000; Whitaker et al., 2007; Whitaker et al., 2010;

Yokoya, 2000; Zlesak et al., 2010) and QTL controlled partial resistance (Carlson-Nilsson, 2000; Korban et al., 1988; Parlevliet, 1981; Roumen, 1994; Shupert, 2005; Whitaker and Hokanson, 2009; Xue and Davidson, 1998) have been characterized.

4.2.4 Marker assisted selection in rose breeding

Compared with selection based on phenotyping only, molecular markers associated with specific traits facilitate plant breeding by identifying the genotypes of potential parents to better design crossing strategies, increasing the speed of selection with young seedling assays and reducing the number of seedlings that need to be phenotyped (Byrne, 2003; Noack, 2003). Besides identifying the desired resistant genotypes, negative selection against unwanted traits may also benefit introgression of new resistance genes from wild species (Debener and Byrne, 2014).

RGAs (resistance gene analogues) and PKs (protein kinase) that are responsible for disease resistance, including powdery mildew and black spot, were characterized and mapped (Hattendorf and Debener, 2007; Linde et al., 2006; Xu et al., 2005; Yan et al., 2005a). For example, the black spot resistance gene *Rdr1* belongs to the class of RGAs with conservative region nucleotide-binding site and leucine-rich repeat (NBS-LRR) (Biber et al., 2009; Kaufmann et al., 2003; Terefe and Debener, 2010; von Malek et al., 2000.). Thus far, there are reports of 3 markers associated with *Rdr1* (Debener and Byrne, 2014; Terefe and Debener, 2010), one associated with *Rdr3* (9.1 cM) (Whitaker et al., 2010) and two markers associated with *Rpp1*, a major gene for powdery mildew race 9 resistance *Rpp1* (Linde et al., 2004).

Although these molecular markers associated with disease resistance could be utilized in MAS as an alternative way of selecting candidate seedlings instead of phenotype based selection only, none of them are utilized in rose resistance breeding programs. Currently the molecular markers are mainly applied on variety and genotype identification, phylogenetic analysis, and analysis and mapping important horticultural traits in rose (Debener et al., 2013).

Like many important commercial characteristics, inheritance of partial resistance is controlled by multiple quantitative trait loci (QTL). The identification of marker-trait associations for QTLs is facilitated by good experimental design and careful phenotyping on hundreds of seedlings for multiple years and/or locations. When heritability is low for those traits, the identification work will be more difficult (Byrne, 2003).

Most recently, important traits controlled by single genes or QTLs could be better characterized by the new generation of molecular marker--the single nucleotide polymorphisms (SNP) marker (Gaj, et al., 2013; Lusser et al., 2012). It is obtained by direct sequencing as an abundant, mainly biallelic, co-dominant marker (Byrne, 2009).

4.2.5 Next generation sequencing and MAS

Next-generation sequencing (NGS) can generate abundant SNP markers with lower cost per marker than previous methods making it an efficient tool for mapping and MAS trait selection in rose breeding (Vera, et al., 2008). NGS can provide re-sequencing data on entire plant genomes or transcriptomes at a greater depth and less cost than standard, fixed-sequence approaches such as single base extension assays or microarrays

(Elshire et al., 2011). The rate of generating DNA sequence data is several orders of magnitude faster than earlier approaches and therefore increases sequencing capacity and makes whole-genome re-sequencing applicable in individual laboratories (Gupta, 2008; Hudson, 2008; Llaca et al., 2012; Mardis, 2008). Unlike the old methods that could only sequence individual genomes, NGS can pool hundreds to thousands of related genomes for sampling genetic diversity within and between germplasm. This approach can be used for the large-scale development of molecular markers for linkage mapping, association mapping, wide crosses and exotic gene introgression, epigenetic modifications, transcript profiling, population genetics and *de novo* genome/organelle genome assembly (Varshney, et al., 2009). Additionally, it can provide the information regarding which fragment of a chromosome is derived from which parent in the progeny line. Consequently, identifying crossover events in every progeny line and placing markers on genetic and physical maps can be done with more confidence (Varshney, et al., 2009).

A current issue is the assembly of whole genome sequence by aligning small fragments without a reference genome. NGS can obtain sequence data from more than one genotype, thereby the alignment could be approached through genome or transcriptome sequence data for model crops that are closely related, or whole transcriptome or reduced representative genome sequence data. Those technologies could provide alignments of short sequences, variants detection and marker discovery, such as developing SNP markers for trait mapping or MAS (Varshney, et al., 2009).

Although NGS has been used to explore *de novo* genome sequencing in several crops already, the cost is still relatively high for sequencing/resequencing and limited more to model plant and major crop species. If the cost for re-sequencing the genome can be reduced to a few hundred US dollars, NGS could be utilized extensively in genome sequencing of parental and progeny lines of mapping populations and the germplasm that are present in different repositories. Additionally, data analysis from large-scale NGS remains a challenge. Mapping the reads to the reference genome is difficult as well because it requires each read to be aligned independently, which leads to the possibility that reads spanning indels could be misaligned (Li et al., 2008; Li et al., 2009; Ning and Mullikin, 2001). Identifying variation from machine artifacts may also result in a high rate and context-specific nature of sequencing errors (DePristo, et al., 2011; Mokry et al., 2010; Wheeler et al., 2008).

Therefore, improvement of tools, pipelines/ platforms are required for efficient, reliable and user-friendly data analysis. For example, several research groups have been making efforts on increasing the accuracy of alignment of NGS because this technology is particularly suited for re-sequencing for SNP generation and variation detection, thereby software that are currently being used tend to be biased toward this application (Smith, 2008). Luckily, some progress has been made such as web-based cyber infrastructure platform Alpheus. This tool is great for pipelining, visualization and analysis of GB-scale sequence data for identification of SNPs and expression analysis (Miller, et al., 2008).

4.2.6 Objectives

The objectives of this study were (1) to screen the broad spectrum of rose germplasm with three molecular markers associated with *Rdr*s to determine if these markers consistently identified roses with the indicated black spot resistance genes, (2) to examine the segregation of *Rdr3* (resistant to race 8) in a cross between a susceptible tetraploid rose ('Golden Gardens') and a resistant triploid rose ('Homerun') with respect to the ploidy of the progeny, (3) to search for potential markers associated with *Rdr3* with bulked segregation analysis conducted on *Rdr3* segregating population 'Golden Garden' (4x) x 'Home Run' (3x) with selected SSRs.

4.3 Materials and methods

4.3.1 Plant materials and molecular markers

To characterize molecular markers on broad spectrum of rose germplasm, two microsatellite markers (155 at 0 cM and 69E24 at 0.1 cM distance) linked to *Rdr1* (resistance to race 3) (Debener, unpublished) were used to screen 208 rose genotypes including TAMU rose breeding materials, the Earth-Kind® collection, Ralph Moore cultivars and various *Rosa* species (Table 14). Twenty-two genotypes have known phenotypes for black spot resistance to race 3 (Zlesak et al., 2010). In addition, one SCAR marker (ND5E) (9.1 cM distance) linked to *Rdr3* (resistance to race 8) (Whitaker, et al., 2010) was used to screen 56 rose genotypes (Table 14). Twenty-five of these genotypes have known phenotypes for black spot resistance to race 8 (Zlesak et al., 2010; current research). The ploidy levels of the rose genotypes ranged from diploid to tetraploid (Zlesak et al., 2010, Ueckert et al., 2014).

To select SSR markers that are associated with *Rdr3*, 38 published markers were utilized in bulk segregant analysis of the progeny of 'Golden Gardens' x 'Homerun' segregating for race 8 resistance (Zlesak et al., 2010) (Table 19). Two DNA bulks, a resistant bulk and a susceptible bulk, were constructed by pooling the DNA of 10 resistant or susceptible individuals. Candidate markers were selected if polymorphism was present from the screening results. These markers were further utilized for screening each individual to calculate the recombination rate and identify any marker tightly linked with *Rdr3*.

4.3.2 Phenotyping of the population 'Golden Gardens' x 'Homerun'

Seven unfolded young leaves (4th-6th nodes from apical of each shoot) from 3 to 5 plants of each seedling were collected for each inoculation. Each side of the leaves was washed with deionized (DI) water for 10 seconds and then placed onto a wet paper towel in a transparent plastic container (152 mm x 140 mm x 59 mm). These were inoculated by spraying them with approximately 2 µL of the conidia suspension (1×10^5 conidia/mL) with asexual conidia of race 8 of *Diplocarpon rosae* that were collected from washing the infected leaves of 'Cl. Pinkie'. After inoculation, the leaves and conidial suspension were incubated for forty-eight hours. Residual water was then removed with a paper towel to avoid possible leaf degradation. The relative humidity in the boxes was maintained at 100% by adding supplemental DI water. The incubation was continued in the lab (~25ºC and 10 h photoperiod) for 14-16 days post inoculation (dpi) at which time the presence of the fruiting structure (acervuli) was checked under the dissecting scope. The individual that developed acervuli, even once, was considered

as susceptible to race 8 of *D. rosae*, otherwise it was categorized as resistant. The entire trial was repeated three times.

4.3.3 DNA extraction

Young leaf tissue (50 mg) was collected from greenhouse and field grown roses and stored at -80°C prior to DNA extraction. DNA was later extracted by using a modified CTAB method (Doyle and Doyle, 1987) (Appendix). After putting approximately 50 mg of leaf tissue in a 1.5 mL microcentrifuge tube, liquid nitrogen was poured into and around the microcentrifuge tube for grinding with a microcentrifuge pestle attached to an electrical drill. 700 µL of 2x CTAB buffer was added to the crushed tissue and the mixture was vigorously vortexed. The homogenate was then placed in a water bath at 65°C for 1 h. Samples were centrifuged at 13,200 gn for 10 minutes and the top aqueous layer was removed and placed in a clean centrifuge tube with 700 µL of CIA added to new tube and inverted several times to mix. This process was repeated three times. The final top aqueous layer was moved into a new microcentrifuge tube containing 500 µL of cold (-20ºC) isopropanol and inverted several times to mix. Samples were stored at -80 ºC for 3 h before centrifuging at 6000 gn for 10 minutes. The supernatant was removed and the pellet of DNA was completely dried out in the tube and subsequently cleaned up by rinsing twice with 70% ethanol. After the ethanol evaporated from the pellet at room temperature, 50 µL of TE was added into the tubes and vortexed for 10 minutes or until completely dissolved. The DNA was quantified with the NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE). A working stock DNA with the concentration of 10 $ng \cdot \mu L^{-1}$ was

created by diluting the sample with nuclease free water. The samples were then stored at -20°C.

4.3.4 PCR amplification

Polymerase Chain Reactions (PCR) were conducted in a 10 µL system including 8 µL of Phusion Flash High-Fidelity PCR Master Mix (New England BioLabs, Inc.), 0.5 µL of each forward and reverse primers (2.5 pmol/µL stock) and 1 µL of DNA (10 ng/µL). PCR cycling was performed on a Benchmark TC9639 Thermal Cycler (Benchmark Scientific, Inc., Edison, NJ) under the following conditions: 10 min initial denaturation at 94ºC, 35 cycles (94 ºC for 30 s, 55 ºC for 45 s, 72°C for 45s), followed by a final extension of 10 min at 72°C. PCR product was later analyzed on a 3.5% MetaPhor agarose gel.

4.4 Results and Discussion

4.4.1 Characterization of molecular markers associated with Rdrs on diverse rose genotypes

SSR markers 155 and 69E24 were scored in 190 and 188 out of 214 genotypes respectively. Among the diverse rose genotypes, 16 of them have known response to race 3 of black spot, in which 4 are resistant and 12 are susceptible (Table 14). The genotypes amplified fragments around 110 bp and 160 bp for the locus 155 and around 180 bp for the locus 69E24. For 155, the detection rate indicated by amplification product at 110 bp is lower (recovered in 3 of 4 resistant roses; 75%) than that of using 160 bp (recovered in all resistant roses; 100%) as an indication fragment. However, the false positive rate was high for both fragments (42-50%). When using both amplification

products as an indication of the presence of *Rdr1*, the detection rate is relatively high (recovered in 3 of 4 resistant roses; 75%), while false positive rate becomes lower (4 recovered in 12 susceptible roses; 33.33%). Thus these markers are not reliable when screening diverse rose genotypes (Table 15, 16).

Although the SSRs 155 and 69E24 were closely linked to *Rdr1* in the population in which they were identified, the presence of these bands was not unique to the plants resistant to the race 3 of the pathogen. This inadequate detection rate and a high false positive detection rate suggested these markers are germplasm specific. Thus they are not useful for the selection for *Rdr1* among a diverse rose germplasm.

Regardless of the plant species and the types of pathogen-host interaction, most plant disease resistance genes contain proteins with conservative structure with a C-terminal leucine-rich repeat (LRR) domain and a central nucleotide binding site (NBS) domain (Jones, 2000). Nine highly similar resistance gene analogues (RGAs) were identified on the contig of *R. multiflora* containing *Rdr1* (Kaufmann et al., 2010). Based on strawberry genome sequence, a few hundred NBS R-genes have been anticipated in rose genome (Bradeen et al., Sixth International Symposium on Rose Research and Cultivation). Therefore race 3 susceptible genotypes may contain other RGA with LRR-NBS conservative region, which are not necessarily related to disease resistance function (Kaufmann et al., 2010). Markers that are flanking in these conservative regions of other RGAs might be the reason for the high false positive rate when screening diverse genotypes by using these two SSR markers since they are closely related with *Rdr1* (0 and 0.1 cM).

Table 14. Genotypes that showed amplification products when screened with markers linked to *Rdr1* (SSR 155 and 69E24) and *Rdr3* (SCAR ND5E). *Rdr1* and *Rdr3* are responding to race 3 and 8 respectively.

Markers	Genotypes screened	Genotypes with amplification	Genotypes with known phenotypes	Resistant Genotypes
SSR 155	214	190	16	4
SSR 69E24	214	188	16	4
SCAR ND5E	51	4	25	5

Table 15. Association of *Rdr1* linked markers SSR 155 and SSR 69E24 amplification products with the resistance for race 3 for 22 rose genotypes.

Genotypes	Ploidy level	Reaction to race 3	Amplification product (bp)		
			155		69E 24
95/13-31(97-7 parent)	2x	R		160	180
Blushing Knock Out	3x	R	110	160	180
Double Knock Out	3x	R	110	160	
Home Run	3x	R	110	160	180
82/78-1(97-7 parent)	2x	S			
April Moon	3x	S	110		180
Belinda's Dream	3x	S	110	160	180
Carefree Marvel	3x	S	110	160	180
Country Dancer	4x	S	110	160	180
Ducher	2x	S			180
Perle d'Or	2x	S		160	180
Prairie Harvest	3x	S			
Quietness	3x	S	110	160	180
Summer Wind	4x	S			
The Fairy	2x	S	110		
Winter Sunset	4x	S			

Table 16. Association of *Rdr3* linked marker SCAR ND5E amplification products with the resistance to race 8 for 25 rose genotypes.

Genotypes	Ploidy level	Reaction to race 8	Amplification product (bp)
Caldwell Pink	2x	R	80
Folksinger	4x	R	80
Homerun	3x	R	
Prairie Harvest	3x	R	80
Quietness	3x	R	
Amiga Mia	4x	S	
April Moon	3x	S	
Belinda's Dream	3x	S	
Blushing Knock Out	3x	S	
Carefree Marvel	4x	S	
Cl. Pinkie		S	
Country Dancer	4x	S	
DD	2x	S	
Double Knock Out	3x	S	
Ducher	2x	S	
FF	2x	S	80
J06-20-14-3	2x	S	
Little Chief	2x	S	
Perle d' Or	2x	S	
R. wichuraiana 'Basye's Thornless'	2x	S	
Red Fairy	2x	S	
Summer Wind	4x	S	
The Fairy	2x	S	
Vineyard Song	2x	S	
Winter Sunset	4x	S	

4.4.2 Phenotype of progenies of GG x HR population

Among the 70 seedlings generated from GG x HR, 27 showed complete resistance to race 8 of *D. rosae* while the rest (43) were susceptible with the presence of acervuli on the leaf tissue. The ploidy level of 56 of the seedlings was determined by counting the chromosomes of root tip cells (Ueckert et al., 2014). Of these, 31 are triploid while 25 are tetraploid. In the triploid seedlings the ratio of resistant and susceptible is 17:14, while in the tetraploid seedlings the ratio was 7:18 (Table 17). From the ploidy level and phenotypes of seedlings, it is clear that the chromosomes of gametes were not randomly assorted.

Table 17. Phenotype of vertical resistance to race 8 (controlled by *Rdr3*) of black spot disease and the ploidy level of seedlings from 'Golden Gardens' x 'Homerun' family. S = susceptible, R = complete resistance.

Seedling #	Phenotype	Ploidy level
7	R	3x
9	R	3x
13	R	3x
14	R	3x
16	R	Aneuploid (21+1)
18	R	4x
19	R	3x
24	R	4x
31	R	3x
32	R	3x
34	R	3x

Table 17. Continued

Seedling #	Phenotype	Ploidy level
35	R	4x
38	R	4x
40	R	?
41	R	3x
42	R	3x
43	R	3x
48	R	3x
50	R	3x
52	R	3x
56	R	3x
57	R	4x
63	R	?
64	R	4x
65	R	4x
68	R	3x
70	R	3x
1	S	3x
2	S	3x
3	S	3x
4	S	4x
5	S	4x
6	S	5x
8	S	4x
10	S	3x
11	S	4x
12	S	3x
15	S	3x
17	S	4x
20	S	4x
21	S	4x
23	S	3x
25	S	3x
26	S	4x
27	S	3x
28	S	4x
29	S	4x
30	S	3x
33	S	3x

Table 17. Continued

Seedling #	Phenotype	Ploidy level
36	S	4x
37	S	4x
39	S	4x
44	S	4x
45	S	3x
46	S	3x
47	S	4x
49	S	4x
51	S	?
53	S	3x
54	S	?
55	S	3x
58	S	4x
59	S	?
60	S	?
61	S	?
62	S	?
66	S	3x
67	S	3x
69	S	4x
71	S	?

Because the seedlings of GG x HR are segregating for *Rdr3*, which conditions complete resistance for race 8, HR should be considered as heterozygous. In addition, due to the existence of tetraploid susceptible seedlings, the donor triploid parent HR most likely only has one copy of the R gene. Although only one third of all tetraploid seedlings are resistant (7 resistant:18 susceptible), slightly more than half of the triploid seedlings are resistant (17 resistant:14 susceptible) (Table 18). It is possible that the frequency of haploid gametes with *Rdr3* is higher or haploid gamete containing *Rdr3* is more favored in fertilization over the diploid gamete containing Rdr3, possibly inherited

from diploid resistant ancestor. Ueckert et al. (2014) discovered that based on the pollen size 1N, 2N, and 3N pollen could be produced by a triploid rose. However, when crossed with a tetraploid female, more seedlings were fertilized with 1N pollen (55%) while 2N pollen fertilized more seedlings when crossed with diploid female parent (75%) (Ueckert et al., 2014). Therefore whether 1N and 2N pollens were evenly distributed by triploid parents remains unclear.

Table 18. Segregation of phenotype of vertical resistance to race 8 of black spot disease and the ploidy level of seedlings from 'Gold Garden' x 'Home Run'. The segregation ratio is tested by Chi-square. S = susceptible, R = complete resistant.

Segregation	Observed	Expected ratio	Chi-square	P-value
R : S	27:43	1:2	0.4	0.87
3x R : 3x S	17:14	1:2	6.7	0.01
4x R : 4x S	7:18	2:1	18.4	0.0001
3x : 4x	31:25	1:1	0.6	0.42

4.4.3 SSR markers associated with Rdr3

Thirty of SSR markers were selected to screen this *Rdr3* segregating population ('Golden Gardens' x 'Home Run') for associations with this target gene (Table 18). The DNA of five resistant and 5 susceptible seedlings were pooled to form the resistant and susceptible bulks. Of the 38 SSR markers used to screen the bulked progeny, only 7 showed polymorphisms between the bulks. These 7 markers were further utilized to screen the entire population with 70 individuals and no marker was associated with *Rdr3* (Table 19). Up to four alleles were amplified from the PCR results and up to seven genotypes were identified at one marker locus. The failure of identifying any closely linked locus flanking with *Rdr3* is probably due to the small number of SSR markers tested. Thus to identify closely linked molecular markers associated with *Rdr3,* more markers (SSRs, SNPs etc.) need to be screened via bulked segregate analysis.

Table 19. Characteristics of the 38 selected microsatellite markers for F_1 population of 'Golden Gardens' x 'Homerun'.

Primer	SSR motif	N of loci amplified	Primer (5′–3′)
69E24[d]		1	F: TCAGGTGGGTGAGCTTCAAT R: TGATTAGCTTGCCGGTTCTT
155[d]		3	F: GAAAAGAACGAGGGGTTTCC R: ACGGTCGGTAATCAAGATGC
Rw1F9[f]	$(ATT)_6$	1	F: GTTGAAGGTAATAAATAACTGAAG R: CAAGGGACGGTAATAAAATC
Rw3K19[f]	$(CAA)_6$	1	F: GCCATCACTAACGCCACTAAA R: GCGTCGTTCGCTTTGTTT
Rw3N19[f]	$(CT)_{20}$	1	F: CTGGCTGGTTCTCTTTCTG R: ATGGGTCGTCGTCGATATG
Rw4E22[f]	$(GA)_2$ $(GAA)_5$	2	F:ATGGGAGACAGAGGTGTAAG R:TCCTAACTCTCGGTGGAGAT
Rw5D11[f]	$(CT)_{14}$	2	F: CAGATTCGCCGTAGCCCTTAC R: ATCCGAACCCCGACCTGAC
Rw8B8[f]	$(TG)_{12}(AG)_{12}$	3	F: GGTAACCAACTTAGCGTTGA R: ATGGCTGCTTCTCTCCTT
Rw10J19[f]	$(GAA)_9$	2	F: GCGAGTTGACGACGAGTT R: GGGTGGGCTTCCTTAGTTA
Rw10M24[f]	$(CT)_7(TA)_4$	1	F: TTAATCCAAGGTCAAAGCTG R: TCTCTTTCCCTCCTCACTCT
Rw11E5[f]	$(CT)_{10}$	1	F: GATACCGCGAAGGTGTAGT R: GAGTGAAAACTCTGCAATCA
Rw12D5[f]	$(CTTT)_2(CTT)_4$	2	F: CCCCTATGCTACACCACAA R: AAGGCTCCAAAGCTTCAC
Rw14A5[f]	$(GAA)_4$	1	F: CCCTCAAAACCCCTCTTA R: CGTAATAACTGTCCGGTCTC
Rw14H21[f]	$(GT)_{16}(GA)_{15}$	1	F: ATCATGTGCAGTCTCCTGGT R: AATTGTGGGCTGGAAATATG
Rw1717[f]	$(GCC)_8(ACC)_3$	1	F: CAGGTAATTTGCGGATGAAG R: GATCCGCCGTTTCCAGT
Rw18N19[f]	$(CTT)_6$	1	F: CCCGAGAAAGAGACAGTAAA R: ATCGAGAGAGACACCGACTC
Rw22A3[f]	$(TTC)_6$	1	F: AGAGAATTGAAAAGGGCAAG R: GAGCAAGCAAGACACTGTAA
Rw22B6[f]	$(CAT)_7$	4	F: ACAGTGAGTTGTTCGCTTCT R: TTCATTGCTAGGAAGCAGTA
Rw25J16[c]	$(TC)_8$	3	F: TGGACCTTCCCTTTGTTTCC R: GCTTGCCCACATATTGTTGA
Rw27A11B[f]	$(AG)_{12}$	0	F: TGTTCCCTTTTAATGAATTAGC R: GTTCATCCCTTCAAACCAC

Table 19. Continued

Primer	SSR motif	N of loci amplified	Primer (5′–3′)
Rw32D19[f]	$(GAA)_7$	1	F: GAAGTCCAGAGCCAATTCCA R: AGGGTCCTCATCCACCACTT
Rw34L6[c]	$(CT)_{16}$	1	F: CTCCTTTAGACTCGGGACCA R: CAGGCACGCCATTTCTAACT
Rw45E24[f]	$(CT)_{45}$	0	F: CAGTTTCATTGCTCGTCTTC R: TATACATGATTCGGGCCTTC
Rw55C6[f]	$(CT)_{11}$	1	F: GTGGATTTTCAGAGATACGC R: TCACAGACAGGACCACCTAT
Rw55D22[f]	$(G)_{12}$	1	F: GATCCGTTTAAGTAACCTTT R: CCACAAGGATTCTGATTTAT
RMS015[c]	(GA)n	0	F: TAATGTAGGCAGATATAAAGGGAGT R: GCAGCTGCACAACAAGGAA
RhE3[b]	$(TGT)_{21}$	1	F: AGATACCCCTTACTT GCATGAATGC R: GTTTCTTGGTTACCTCCAAAACCAGAAACC
H22C01[c]	$(TC)_9$	1	F: TCATAACCAACCATCTCCATCA R: AGGATTTCACCCAGAACACG
H23O17[c]	$(CT)_{11}$	1	F: ACACCAAGCAAACCAAAACC R: AGCACGAAAACCGAGAGAGA
H24D11[c]	$(CT)_{10}$	1	F: CCTCCTCAGCTTTCCTCCTT R: CAGCAACCATCTCTTCGTGA
CL3881[c]	$(TTTG)_4$	2	F: GACAACGACCACACCACTTG R: CCAAAGCAACATTGTCAAAAGA
RhAB9-2[b]		3	F: GTCAATTTGTGCATAAGCTC R: GTGAGAACAGATGAGAAATG
Rh58[e]		4	F: ACCAATTTAGTGCGGATAGAACAAC R: GGAAAGCCCGAAAGCGTAAGC
RhD201[a]	$(TCT)_{33}$	2	
RhE3[b]	$(TGT)_{21}$	3	F: AGATACCCCTTACTT GCATGAATGC R: GTTTCTTGGTTACCTCCAAAACCAGAAACC
RhI402[b]	$(GTG)_{11}$	3	F: TCCCATCTTGCTAAG TGCCTT R: GTTTCTTCAGGGTAACTGAGCCGATT
H20D08[c]	$(CT)_{10}$	2	F: TTCGGCTCTCTTCTCTGCTC R: GACATTACAGCGACGAAGCA
RhO517[b]	$(GAC)_7$	1	F: CGGCGACGAACA AATCAGCATATC R: GTTTCTTTGAAGAACGAGGCGCAGCGTAA

[a, b, c, d, e, f], characteristics of marker can be referred to Debener et al., 2001, Esselink et al., 2003, Oyant et al., 2008, Whitaker et al., 2010, Yan et al., 2005, and Zhang et al., 2006.

Table 20. Characteristics of the 7 selected microsatellite markers for F_1 seedlings of 'Golden Gardens' x 'Homerun'. R = resistance. S = susceptible.

	Bulk Analysis		Polymorphism			
Primer	**R**	**S**	**Resistant progeny**	**N° Amplified seedlings**	**Susceptible progeny**	**N° Amplified seedlings**
Rw8B8	ac	abcd	c	3	c	5
			ab	1		
			ac	1		
			bc	4	bc	17
			abc	13	abc	16
			bcd	1		
			abcd	3	abcd	3
Rw22B6	ac	bcd	c	1	c	10
			ac	1		
					bc	6
			cd	10	cd	9
			abc	4	abc	9
			bcd	3	bcd	5
			abcd	10	abcd	3
RhAB9-2	abc	bc	a	6	a	6
			b	1	b	4
			c	2		
					ab	5
			ac	9	ac	14
			bc	3	bc	11
			abc	4	abc	1
Rh58	ac	abcd	c	1	c	1
			ad	3	ab	1
					ac	3
					ad	7
					bc	1
			cd	3	cd	1
			acd	9	acd	13
			bcd	2	bcd	2
			abcd	8	abcd	3
RhE3	a	abc	bc	14	bc	18
			abc	13	abc	24
RhI402	ab	abc	bc	8	bc	12
			abc	13	abc	20
			ac	5	ac	7
				26	c	1
H20D08	ab	b	ab	12	ab	19
			b	15	b	24

CHAPTER V

CONCLUSION

The work in this dissertation examined the inheritance of partial (horizontal) resistance and the markers associated with complete (vertical) resistance to black spot in roses.

Two artificial inoculation methods, detached leaf assay (DLA) and whole plant inoculation (WPI) were conducted on breeding materials in Chapter II. No complete resistance to race 8 controlled by single dominant gene *Rdr3* was detected in our diploid germplasm. A wide range of partial resistance was observed and the performance of different roses could be distinguished by DLA and WPI. As disease development measured by DLA and WPI was highly correlated ($R > 0.8$), only DLA was utilized for phenotyping for subsequent studies because it allowed for the maintenance of optimal conditions for pathogen growth and is adaptable for large scale phenotyping.

A partial diallel was constructed by intercrossing resistant breeding lines with moderately resistant and susceptible roses. Progenies from hybrid diploid populations were phenotyped to characterize partial resistance to black spot disease (Chapter III) with DLA using both LL and LAS to assess the relative black spot resistance of the rose genotypes. The variance analysis of the transformed data (square root) indicated that 24%-34% (LAS and LL) of the genetic variance of partial resistance was explained by additive variance. In contrast, the narrow sense heritability, as calculated by the offspring mid-parent regression approach ranged from 0.74-0.86. This indicates that

partial resistance as measured by DLA is a moderately to highly heritable trait. For field data collected in the trial, the narrow sense heritability estimated from genetic variances of combined S13 and F13 was very similar (0.3) to that of DLA (0.3-0.4) and both lower than the estimation from offspring mid-parent regression (0.74-0.86), therefore partial resistance can still be considered as a heritable trait. High non-additive variance in DLA (explained approximately 50%-60% of total genetic variances) suggested selection among families before selecting elite seedlings in those populations. However, high narrow sense heritability estimated from field data and offspring mid-parent regression (0.74-0.88) indicated stronger additive effects than non-additive effects of partial resistance trait. Therefore, both within populations and among populations selections were made when advancing elite seedlings for further research with most of them coming from J14-3 x VS and J4-6 x RF.

Although field assessment is the most commonly used method for selecting candidate seedlings in a rose breeding program, it is time consuming (2-3 years) and may be inconsistent due to the variation of climate and disease pressure. Evaluations conducted during the late fall in Texas were more reliable due to the more optimal environmental conditions (cooler temperatures and more precipitation) for pathogen development which lead to higher inoculum levels. Field assessments could be improved by increased and more uniform inoculation in field trial such as by planting new rows next to an established trial already infected with the disease and by planting susceptible individuals randomly in the trial (Debener and Byrne, 2014). More measurement components such as defoliation could be utilized during field assessment as well to

better correlate with DLA because research showed that LL from DLA correlated with defoliation rating from a 2-year field assessment (R = 0.618) but inversely correlated with overall performance rating (R = -0.642) (Zlesak et al., 2010). It is possible that the pathogen infection triggers defoliation on living plants, while on detached leaves successful infection leads to better mycelia development. DLA, as an alternative evaluation tool provides consistently optimal conditions for disease development and a well-defined pathogen by using single spore cultures. A low (r = 0.1-0.2) correlation was detected among fall field assessment results from 2012-2013 and DLA possibly due to (1) only one cycle of disease development is allowed in DLA whereas multiple cycles occur in the field, (2) measurement components utilized in the field does not characterize the same aspects of disease development as DLA, (3) multiple disease resistance mechanisms may occur on the host plant in the field triggered by multiple races, and (4) other diseases such as cercospora may cause confusion in field assessment since they have similar symptoms.

Within DLA, the lesion length and lesion size measurement were highly correlated (R=0.9) when estimating among the parental materials but much lower (R=0.3 or 0.2) when using data from the segregating progenies. A possible reason for this would be the greater range of LL among the parental materials (0.1-7.14mm) as compared to the progeny materials (ranging from 0.5-3.0 mm and 0.5-2.4 mm).

In rose breeding, especially for trait introgression, molecular markers associated with the target traits could be an efficient tool to identify candidate genotypes, to select extreme seedlings to reduce the amount of seedlings for phenotyping, and/or negatively

select against unwanted traits during introgression (Byrne, 2003; Noack, 2003; Hosseini Moghaddam et al., 2012; Debener and Byrne, 2014). However, markers associated with *Rdr*s (*Rdr1* and *Rdr3*), seemed only effective on the germplasm in which they were generated, while in the case of *Rdr3* a loose linkage might be an additional reason of poor correspondence between the marker and resistance.

The transmission of *Rdr3* from the triploid cultivar 'Homerun' when crossed with the black susceptible tetraploid 'Golden Gardens' was non random and differed with the ploidy of the seedlings. Due to the lack knowledge on the distribution of haploid and diploid gametes of 'Homerun', transmission and assortment of the chromosome containing *Rdr3* remains unclear. Initial work to find an SSR associated with *Rdr3* did not reveal any marker-trait associations. Further work needs to be done with more markers (SSRs, SNPS, etc.).

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APPENDIX

STOCK SOLUTION PREPARATIONS FOR DNA EXTRACTION

2X CTAB buffer (100 ml):

2% CTAB - 2.00 g
1.4 M NaCl - 8.12 g
20 mM EDTA, pH 8.0 - 4 ml of 0.5 M
100 mM Tris HCl, pH 8.0 - 10 ml of 1.0 M
1% PVP-40 (polyvinylpyrollidone, M.W. 40,000) - 1.00 g
β-Mercaptoethanol - 200 µL

Note: CTAB is difficult to dissolve. Do not add β-Mercaptoethanol until ready to use.

0.5M EDTA, pH 8 (1000 ml):

EDTA (Disodium ethylenediaminetetraacetate·$2H_2O$) - 186.1 g

Preparation: Add 186.1 g of EDTA to 200 mL of water. Stir vigorously on a magnetic stirrer. Adjust the pH to 8 with NaOH (~20 g of NaOH pellets), then adjust volume of the solution to 1000 mL with water.

Note: EDTA will not go into solution until the pH of the solution is adjusted to approximately 8 by the addition of NaOH.

1.0 M Tris HCl, pH 8 (1000 ml):

Tris (Hydroxymethyl) Aminomethane - 121.14 g

Preparation: Dissolve 121.14 g of Tris in 800 mL of water. Adjust the pH to 8 by adding HCl (~42 mL of concentrated HCl). Allow the solution to cool to room temperature before making final adjustment to the pH. Adjust volume of the solution to 1000 mL with water.

TE (100 mL):

10 mM Tris·HCl - 1.0 mL of 1.0 M
1 mM EDTA - 0.5 mL of 0.5 M

Note: Bring solution to 100 mL with nanopure water.

CIA (100 mL):

Chloroform - 96 mL
Isoamyl Alcohol - 4 mL

Note: Store CIA at -20°C.